HOLSTEIN HEIFER BEHAVIORS AND LACTATING COW NUTRITIONAL MANAGEMENT FOR IMPROVED REPRODUCTIVE PERFORMANCE ON DAIRY FARMS

BY

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THESIS

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ABSTRACT

As the industry pushes for more productive cows, the reproductive performance on farms declines. Poor reproductive management on farms can be costly due to decreased milk production in late lactation, higher feed costs from a longer calving interval, and more labor and supplies from multiple breedings. To investigate techniques to improve the reproductive efficiency on dairy farms, two experiments were conducted involving Holstein heifers of breeding age and early postpartum Holstein cows.

To investigate tail paint utilization and potential relationships among behaviors and activity, eighteen Holstein heifers were randomly assigned to one of three treatments: control (**CON**), tail chalk; treatment-A (**TRTA**), tail chalk with proprietary ingredient; and treatment-B (**SPRAY**), spray formulation. Experimental design was a replicated 3x3 Latin square with 6 total squares, 3 animals per square, and 3 periods of 14 d each. Visual observations were performed for thirteen behaviors in 30 min segments every 2 h from 6 AM to 6 PM. A synchronization protocol starting on d 1 of each period (Ovsynch®: 100mg GnRH at d 1, 25mg PGF2α at d 7, and 48 h later an application of 100mg of GnRH) was used to stimulate periods of high and low interactions. Ovaries were examined via ultrasound imaging on d 1, 8, and 10 of each period. Statistical analyses were performed using the GLIMMIX and MIXED procedures of SAS (v9.4). Heifers receiving SPRAY had lower number of licks received per day ($P < 0.001$) and lower tail paint removed regardless of day or follicle size ($P < 0.01$) when compared with CON or TRTA. Rump lick received ($P < 0.01$), chin rest received ($P < 0.01$), anogenital sniff received ($P = 0.02$), mount received ($P < 0.01$), and both initiated and received behaviors for attempt to mount ($P < 0.03$) occurred more in heifers with large follicles on d 8 and d 10. Dairy operations that have problems with tail paint removal and false-positives may benefit from changing to a tail

paint product with a different consistency, such as a spray formulation. Producers looking for heifers to breed should focus on those receiving rump lick, chin resting, anogenital sniff, mount, and attempt to mount, or increases in daily activity.

Next we examined the effects of rumen-protected methionine or choline supplementation on uterine health. Seventy-two Holstein cows were fed the same TMR and randomly assigned to four treatments from calving to 30 DIM. Treatments were: **CON** (n = 16, fed TMR with a Lys:Met = 3.5:1), **MET** (n = 20, TMR + Smartamine M® to a Lys:Met = 2.9:1), **CHO** (n = 16, TMR + 60 g/d Reashure®), and **MIX** (n = 19; TMR Smartamine M® to a Lys:Met = 2.9:1 and 60 g/d Reashure®). Starting at d 31 cows were randomly re-assigned to two treatments: (**CON**; n = 36, TMR with a Lys:Met = 3.4:1) or (**MET**; n = 36, TMR + Smartamine M® to a Lys:Met = 2.9:1). Cows were evaluated at 4, 7, 10, 13, 15, 17, and 30 d after calving for the presence of secretion using the Metricheck® device. Contents were scored at 0, 1, 2, or 3 and smell was scored at 0 or 3. On 15, 30, and 72 d after calving, the uterine endometrium of all cows was sampled using a cytological brush and streaked onto slides. Each slide was counted by one person for the presence of polymorphonuclear neutrophils (**PMN**). Statistical analysis was performed using the MIXED procedure of SAS. On d 30, a treatment difference was detected using the metricheck score and smell ($P < 0.04$), with treatment MIX (0.38) having a lower score than CHO (2.11). In conclusion, supplementing cows with rumen-protected methionine may have a beneficial effect on cows' uterine health. The use and combination of techniques in this thesis may improve reproductive performance across dairy farms and have a huge impact in profitability.

LIST OF ABBREVIATIONS

AA: Amino acid

AFC: Age to first calving

AI: Artificial insemination

BCS: Body condition score

BW: Body weight

CHO: Treatment of supplemented choline from Chapter 3

CON: Control treatment

DA: Displaced abomasum

DAb: Double antibody kit for assays

DMI: Dry matter intake

EIA: Enzyme-linked immunoassay

E2: Estradiol

MC: Metricheck

MET: Treatment of supplemented methionine from Chapter 3

MIX: Treatment of supplemented methionine and choline from Chapter 3

NEB: Negative energy balance

NEFA: Nonesterified fatty acid

PBS: Phosphate-buffer solution

PC: Phosphatidylcholine

PMN: Polymorphonuclear leukocytes

P4: Progesterone

RIA: Radioimmunoassay

RP: Retained placenta

SAb: Single antibody kit for assays

SAS: Statistical analysis software

SPRAY: Treatment-B from Chapter 2

TMR: Total mixed ration

TPR: Tail paint removed variable from Chapter 2

TRTA: Treatment-A from Chapter 2

VLDL: Very-low density lipoprotein

For my husband,

Vernon W. Skenandore II,

For always supporting me and being my best friend

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INTRODUCTION

Through intensive management practices and progress in genetics, the dairy industry has been rapidly changing over the years. To meet the increasing demand for a growing population, smaller numbers of cows are producing more milk (USDA census data, 2012). Trends also show a decrease in the number of farms, but an increase in herd size per farm (USDA census data, 2012). These changes have created many new challenges in the industry, one of which is the decline in fertility and reproductive efficiency.

This decline is not a recent phenomenon. Decreases in reproductive measures can be dated to the mid 1900's (Lucy, 2001). For example, Butler (1998) reported a decline in first-service conception rates from 65% in 1951 to 40% in 1996. Other studies have shown increasing services per conception, days open, and days to first insemination (Lucy, 2001). There has been a long history of associating increased milk production with decreased reproductive performance in dairy cattle, but with high-producing herds sometimes having better reproduction, other changes in the industry may have equivalent or greater effects on reproduction (Lucy, 2001). Improved reproduction in high-producing herds most likely reflects better feeding, healthier cows, and better reproductive management (Lucy, 2001). For example, larger farms are now having to adjust management practices that were meant for traditional, small farms. Since there are a greater number of cows per farm, larger farms require more time for heat detection, identification, insemination, and record keeping (Lucy, 2001). Reproduction is also greatly affected by health. Cows in confinement and in larger herds have increased risk of mammary and uterine infections (Goldberg et al., 1992; Kaneene and Miller, 1994), which are risk factors for infertility. If cows are housed in larger confinement herds and have a higher incidence of disease, it follows that they will have decreased fertility (Lucy, 2001).

The reproductive efficiency of dairy cows greatly impacts the profitability of farms (Plaizier et al., 1997; Meadows et al., 2005) and reproductive performance is often a major reason for premature culling of dairy cows (Beever, 2006). As reproductive efficiency declines, profits can be affected by reduced milk production, increased reproductive culling, fewer replacement heifers, increased semen costs, and added veterinary costs (Britt, 1985). Milk production is contingent on the ability of cows to become pregnant, since lactation is stimulated by calving. For the greatest efficiency and lifetime productivity, heifers must become pregnant at an optimal age and cows must become pregnant while still in lactation. Studies have shown that each day cows and heifers are not pregnant beyond the optimal time can be very costly (Holmann et al. 1984; Tozer and Heinrichs, 2001; Groenedaal et al., 2004, Meadows et al., 2005). These costs are most likely from non-producing heifers or an increased time in late-lactation, where cows produce less and less milk until they are dried off. If animals are not inseminated and become pregnant in a timely manner, they will spend more time producing less milk. With feed being the greatest expenditure on dairy farms, it is more profitable to feed cows that produce more milk, rather than heifers, and to limit the days cows are in late-lactation.

Increasing reproductive efficiency will increase profits on dairy farms. Therefore, the objectives of my thesis are as follows:

1. To better understand estrus detection in heifers by comparing the behaviors associated with 3 different types of tail paint formulations in Holstein heifers. (Chapter 2).
2. To determine the effects of feeding rumen-protected methionine and choline to postpartum Holstein cows on reproduction through the assessment of vaginal discharge and uterine cytology (Chapter 3).

CHAPTER 1

LITERATURE REVIEW

HEIFERS

Dairy heifers are the future of a dairy operation, but are also a significant source of cost. The total cost of raising heifers has been shown as the second largest contributor to operating costs in Pennsylvania and the third largest in Michigan (Harsh et al., 2001; Tozer and Heinrichs, 2001). The costs represent 12.5 to 20% of the total annual expenses on dairy farms (Karszes, 1994; Harsh et al., 2001). The largest component of heifer expenses, like with dairy cows, is feed costs that account for nearly 73% of the total from birth to calving (Heinrichs et al., 2013). Because heifers are in a pre-productive stage, many operations fail to see the importance of proper heifer management. Heifer management decisions can have a compound effect on current and future farm profitability in the form of hidden expenses and lost productivity (Zanton and Heinrichs, 2005).

The age at first calving (**AFC**) is one of the most important factors when working with heifers. For optimal milk production and rearing costs, it is recommended that the AFC for dairy heifers is 23 to 24 months and the body weight is > 560 kg after calving (Heinrichs, 1993). Unfortunately, only 2.7% of Holstein dairy farms in the United States actually meet the recommend target (Losinger and Heinrichs, 1997). The AFC is usually manipulated by altering growth rates through nutrition (Van Amburgh et al., 1998). However, even when heifers are managed to achieve similar growth rates, variability on the AFC is observed due to differences in reproductive efficiency (Ettema and Santos, 2004). Reduced AFC not only offers advantages to management practices, but also decreased feed costs, decreased overcrowding, and increased production per day of herd life (Goodger et al., 1989). There was an estimated decrease in

rearing costs of 18% when the AFC decreased from 25 to 21 mo (Tozer and Heinrichs, 2001). In order to decrease variability and meet the optimal recommended AFC, it is best to achieve high pregnancy rates through great reproductive management.

The most effective way to achieve high conception rates in dairy heifers is to breed them while they are in standing estrus, a period of time immediately before ovulation where heifers will stand to be mounted by others. Ovsynch® was the first protocol developed to successfully synchronize ovulation in lactating dairy cows, which allowed for timed artificial insemination (**AI**). Unfortunately, dairy heifers respond poorly to Ovsynch® and yield conception rates 20 to 40% lower than heifers receiving AI from estrus detection (Schmitt et al., 1996; Pursley et al., 1997). Poor response and costs incurred from hormones and labor make Ovsynch® and other protocols a poor choice for heifers. Thus, visual estrus detection remains the most effective way to breed heifers in estrus. The disadvantage here is that it can be time consuming and costly to properly train herdsmen. A study done by Pelissier (1976) showed that estrus detection failure was the number one cause of delayed first service and contributed to the delay of subsequent services.

Estrus detection aids were developed to assist visual observations of estrus and have long been used (Foote, 1975). Especially since herds have expanded to such large sizes, traditional methods meant for small farms have served the modern dairy industry poorly (Lucy, 2001). Larger herds simply require more time for estrus detection and can benefit from using aids such as tail paint and activity monitors. Time saved from the use of detection aids also cuts down on labor costs.

TRANSITION PERIOD AND METABOLIC DISORDERS

Once pregnant, heifers and cows must prepare for the transition period and subsequent pregnancies. The transition period is usually identified as the 3 wk before and 3 wk after parturition (Drackley, 1999). This is a critical time where most of the infectious diseases and metabolic disorders occur (Drackley, 1999). Various endocrine changes, such as high estrogen in the blood, are contributing factors to decreased dry matter intake (**DMI**) in the transition period (Grummer, 1993). This decrease causes fat mobilization and an increase in nonesterified fatty acids (**NEFA**). These problems increase with increasing negative energy balance (**NEB**); when the energy demand for lactation and maintenance exceed the energy intake (Bauman and Currie, 1980). Normal liver function can be affected as fat accumulates in the liver, leading to a disorder known as fatty liver. This happens when there is not enough synthesis of very low density lipoproteins to help export fat away from the liver. As fat accumulates, the ability for the liver to convert propionate to glucose is impaired (Overton et al., 1999). The NEFA in the liver are then converted to acyl-CoA, which is oxidized to acetyl-CoA, and further oxidized to energy for the TCA cycle. If the TCA cycle is challenged, acetyl-CoA can be used for the synthesis of ketone bodies (Drackley, 1999). These ketone bodies can be sources of fuel for other tissues when glucose is low (Leslie et al., 2000). However, with a great increase in glucose utilization (especially in the mammary gland) at the onset of lactation and the decrease in DMI, there is a risk of fat mobilization exceeding the rate of ketone utilization. If this happens, ketone bodies can accumulate in the blood, leading to ketosis, and have detrimental effects on the health and productivity of cows (Ingvartsen and Anderson, 2000; Drackley et al., 2001). In addition, because the DMI is decreased, there is lower intake of nutrients essential for the immune system, therefore contributing to immunosuppression. Hypocalcemia, low concentrations of calcium in

the blood postpartum from the onset of milk synthesis, may also compromise the function of immune cells (Kimura et al., 2006). With the immune system compromised, the lymphocyte and neutrophil functions are impaired (Goff and Horst, 1997).

NUTRITION AND REPRODUCTION

The impact of nutrition on reproduction has been well documented (Robinson, 1996; Boland et al., 2001; Robinson et al., 2006). Challenges of the metabolism during the transition period may have direct or indirect influences on fertility, and difficult transitions have negative impacts on subsequent reproduction (Chapinal et al., 2012). Infertility in dairy cows has been linked to NEB (Jorritsma et al., 2003), which is influenced heavily by DMI. The variation in NEB has been shown to be more heavily influence by DMI ($r = 0.73$) and less by milk yield ($r = -0.25$) postpartum (Villa-Godoy et al., 1998). Therefore, higher producing cows do not necessarily have greater NEB compared to lower-producing herd mates. When DMI is decreased around calving, cows are forced to make decisions about where to direct the scarce nutrients, and early postpartum nutrients will be directed more for immediate survival in milk production rather than to reproduction and the next pregnancy (Friggens, 2003).

Disorders associated with postpartum NEB, like fatty liver and ketosis, are associated with impaired reproductive performance (Rukkwamsuk et al., 1999; Jorritsma et al., 2003; Walsh et al., 2007; McArt et al., 2012). Postpartum NEB can also cause a decrease in body condition score (**BCS**). Cows that lost > 1 BCS (on a scale of 1 to 5) had greater incidence of metritis, retained placenta (**RP**: placenta that failed to completely deliver longer than 12 h after calving), milk fever, ketosis, displaced abomasum (**DA**), and a longer interval to first breeding (Kim and Suh, 2003). More so, cows that develop clinical hypocalcemia are 3.2 times more likely to experience a RP (Curtis et al., 1983), and hypocalcemia has been linked to uterine disease

(Whiteford and Sheldon, 2005). It has also been reported that increased NEFA and RP early postpartum are major risk factors for metritis (Dubuc et al., 2010a). Retained placenta and metritis are diseases from impaired immune function and can have long lasting negative effects on uterine health and fertility, such as reduced conception rates and extended intervals to pregnancy (Goshen and Shpiegel, 2006; LeBlanc, 2008). Furthermore, metritis, low BCS, and hyperketonemia are risk factors for endometritis (Dubuc et al., 2010a; Cheong et al., 2011). Since the depth and duration of NEB are highly related to DMI (Zurek et al., 1995; Drackley et al., 2005), reproductive problems may be mitigated by minimizing postpartum disorders and maximizing the DMI.

One technique that has been recently used around parturition has been to supplement amino acids (**AA**). During the transition period, AA are needed to help synthesize phosphatidylcholine (**PC**), which is important in the formation of VLDL. As mentioned before, VLDL play a role in exporting fat from the liver. Some AA, such as methionine, can also play a part in better oxidative and immune function (Durand et al., 1992; Chen et al., 2007). Methionine has also been shown to increase DMI (Pisulewski et al., 1996; Ordway et al., 2009; Osorio et al., 2013). However, lysine and methionine are the two most limiting amino acids due to their increased presence in milk (NRC, 2001). Sources of lysine in the USA are easy to find, such as blood meal, but sources of methionine in dairy cow diets can be more difficult to incorporate. It is important to adequately feed methionine because it is an important methyl donor and plays a part in many metabolic processes. For example, methionine helps form cysteine for glutathione metabolism (an antioxidant) and can be converted to S-Adensyl-Met (SAM) for DNA methylation and PC (Figure 1.1). Fortunately, an alternative to forming PC is to use choline as a precursor. Choline supplementation before and after calving has been shown to reduce fatty liver

and incidence of ketosis and mastitis (Lima et al., 2012). Increasing DMI and reducing the risk of metabolic disorders by improving liver function and increasing immune function in the transition period is key to better reproductive health.

UTERINE HEALTH

It is important to properly define and diagnose uterine infections so that treatment can be applied and prevention techniques adapted for future lactations. Unfortunately there is no gold standard for diagnoses and there are many different terms and definitions that are often used interchangeably. Therefore, it is important to look at each uterine disease, the definition and terminology, and techniques to diagnose.

Metritis is an obvious clinical disease and is quickly diagnosed. If conducting a full physical exam, one can diagnose *puerperal metritis* within the first 21 d after parturition. Puerperal metritis is most common in the first week and is characterized by a fetid, red-brown watery uterine discharge, associated with clinical signs such a decreased milk yield and a fever of > 39.5ºC (Sheldon et al., 2006). If animals are not ill (not showing clinical signs of illness) but do have purulent (> 50% pus) discharge detectable in the vagina within 21 d after parturition, they would be diagnosed with clinical metritis, or more commonly, *metritis* (Sheldon et al., 2006). The incidence of metritis can range from 8 to > 40% on farms (Galvão, 2013). This disease can be diagnosed by a vaginoscope, ultrasound, a clean gloved hand, or a Metricheck® device.

The vaginoscope is usually performed by inserting a lubricated speculum into the vagina until the cervix can be visualized and then characterizing the contents with the assistance of a small light (McDougall et al., 2007). However, the vaginoscope is an uncommon diagnostic tool because it is perceived to be inconvenient by clinicians, has higher cost, potential for disease

transmission, and has lower sensitivity than other tools (LeBlanc et al., 2002; Sheldon et al., 2006; McDougall et al., 2007). Transrectal ultrasonography permits specific measurements of the reproductive tract, but does not provide any more information about metritis than the examination of the contents and can be more time consuming (Sheldon et al., 2006). The most simply method of examining contents of the vagina is to withdraw them from the animal. The advantage here is the technique is quick, inexpensive, and provides extra sensory information such as smell (Sheldon et al., 2006). Two types of withdraw techniques are with a clean, gloved hand and a Metricheck® device. The gloved hand technique is usually done for < 30 s at a time and involves palpating the dorsal and ventral walls of the vagina (Sheldon et al., 2006). This technique has been validated and does not cause uterine bacterial contamination or delay uterine involution, but there may be an increase discomfort to the cow with larger hand sizes (Sheldon et al., 2006). A somewhat new, validated approach to examining vaginal contents is with the Metricheck® (**MC**; Pleticha et al., 2009). This device consists of a 50 cm long stainless steel rod with a 4 cm rubber hemisphere tip that is used to collect vaginal contents. The MC is inserted through vulva and into the cranial portion of the vagina fornix, after which the tool is retracted at a slight upward angle to not lose any contents. This technique is quick, inexpensive, sanitary, and easy to use. Once contents are collected but either the gloved hand or MC, they can be examined and scored on a scale of 0 – 3: score 0 = clear or translucent mucus; score 1 = mucus containing small flecks of white or off-white pus; score 2 = discharge containing ≤ 50% white or off-white mucopurulent material; and score 3 = discharge containing ≥ 50% purulent material, usually white or yellow, but sometimes sanguineous (Sheldon et al., 2006). Contents are also smelled and quantified (smell 0 = no odor or smell 3 = fetid odor).

After 21 d postpartum a different type of uterine disease is diagnosed called endometritis. There are two types of endometritis that are mostly based on the diagnostic technique: clinical and subclinical. *Clinical endometritis* is not associated with systemic signs of illness and is characterized by the presence of purulent discharge 21 d or more postpartum or mucopurulent (≥ 50% pus and 50% mucus) discharge 26 d or more postpartum detected in the vagina (Sheldon et al., 2006). Clinical endometritis affects about 5 to >30% of cows on some farms and is diagnosed with the same techniques as metritis (Galvão, 2013). *Subclinical endometritis* is endometrial inflammation of the uterus and has to be diagnosed through cytology. Subclinical endometritis is defined as >18% neutrophils 21 to 33 d or > 10% neutrophils at 33 to 47 d postpartum, in the absence of clinical endometritis (Sheldon et al., 2006). The neutrophils are known as polymorphonuclear (**PMN**) leukocytes for their multi-lobed nucleus. These cells make up to 70% of the circulating white blood cells (Goldsby et al., 2000) and are the first response of innate immunity in the uterus at parturition. Subclinical metritis is the most prevalent of all uterine diseases with an incidence of 11 to 70% in some herds (Galvão, 2013). It is usually diagnosed with techniques such as a cytology brush, uterine lavage, or uterine biopsy.

Uterine biopsies were used initially for the study of infertility in mares (Chapwanya et al., 2010). Biopsy samples are taken from cows by manipulating a small biopsy gun through the cervix and extracting a small piece of the uterine wall from the uterine body. Biopsy samples are rarely used for the use of diagnosing uterine diseases because it is time consuming, expensive, invasive, and can negatively impact fertility if done incorrectly (Kasimanickam et al., 2005; Sheldon et al., 2006; Dubuc et al., 2010b). In addition, biopsy samples do not agree with cytology samples for the diagnoses of endometritis (Madoz et al., 2014). Endometrial cytobrush and low-volume uterine lavage are the most common techniques

(Kasimanickam et al., 2004; Gilbert et al., 2005). Uterine lavage is done by inserting a catheter into the uterine body, flushing a small volume of media into the uterus, and subsequently collecting the fluid to make future cytology samples (Gilbert et al., 2005). However, some fluid can produce endometrial irritation and the lavage technique increases time required to obtain samples, increases the distortion of cells harvested, and causes a 17% of failure in attempts to recover fluid (Brook, 1993; Kasimanickam et al., 2005). Therefore, the cytology brush is becoming an increasingly popular technique to diagnose subclinical endometritis. The use of a cytology brush is less harmful than the either techniques and has also been performed in the mare (Defontis et al., 2011). This technique is done by inserting a cytology brush into a sterile stainless steel rod and then placed into a stainless steel tube for passage through the cervix. The tube is then placed in a sanitary plastic sleeve to prevent contamination. The instrument is passed into the cervix and advanced into the body of the uterus. In the uterine body, the stainless steel tube is pulled back to expose the cytology brush. Endometrial samples are collected by rotating the handle of the stylet while in contact with the uterine wall. The cytology brush is then retracted back into the stainless steel tube prior to removal from the cow. Slides are then made immediately following the sample collection and the percentage of PMN is counted using a microscope or pathology software.

CONCLUSIONS

Heifers are no exception to problems in reproductive efficiency. Better detection of estrus through visual observation or the use of detection aids can improve reproductive efficiency on dairy farms. Improving reproduction by decreasing the AFC will save money from feed costs and missed production. Furthermore, the transition period is a critical time for dairy cows. Research has suggested that inadequate DMI can lead to serious problems such as decreased

immune function, greater NEB, increased fat mobilization, and a higher risk of uterine disease. These problems are also related subsequently to fertility. Therefore, nutritional and management strategies that optimize DMI and minimize lipid mobilization around parturition should also improve uterine health and fertility.

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FIGURE

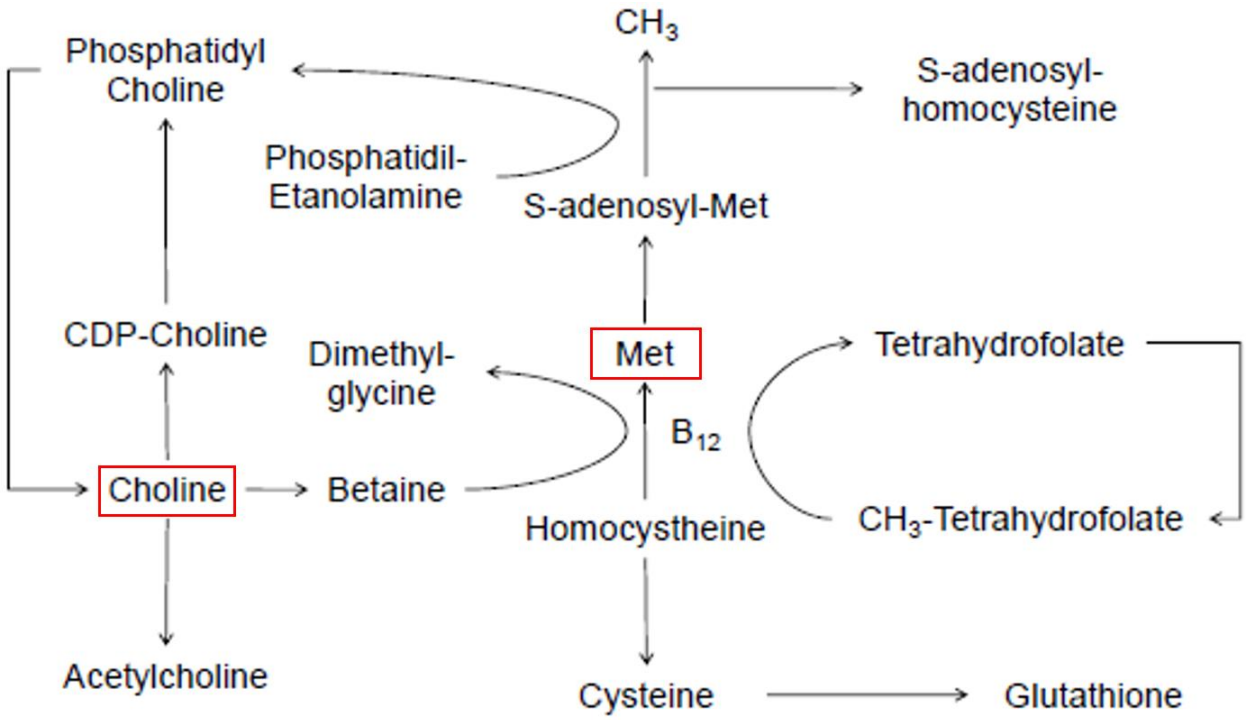

Figure 1.1. Methionine and choline pathway

CHAPTER 2

COMPARISON OF THREE TAIL PAINT FORMULATIONS ON BEHAVIOR OF HOLSTEIN HEIFERS

ABSTRACT

To investigate tail paint utilization and potential relationships among behaviors and activity, eighteen Holstein heifers were balanced by age (13.7 ± 1.2 mo), body weight (394 ± 32 Kg), and body condition score (3.43 ± 0.1 on a 1 to 5 scale), and randomly assigned to one of three treatments. Experimental treatments were commercial formulations and orange in color: control (**CON**), tail chalk; treatment-A (**TRTA**), tail chalk with proprietary ingredient; and treatment-B (**SPRAY**), spray formulation. Experimental design was a replicated 3x3 Latin square with 6 total squares, 3 animals per square, and 3 periods of 14 d each. Visual observations were performed in 30 min segments every 2 h from 6 AM to 6 PM. Thirteen behaviors were recorded (social lick, rump lick, tail paint lick, body butt, head butt, chase up, push, chin rest, anogenital sniff, play rub, winner, mount, and attempt mount). A synchronization protocol starting on d 1 of each period (Ovsynch: 100mg GnRH at d 1, 25mg PGF2α at d 7, and 48 h later an application of 100mg of GnRH) was used to stimulate periods of high and low interactions. Ovaries were examined via ultrasound imaging on d 1, 8, and 10 of each period. The presence of follicles or a corpus luteum (CL) was recorded with their respective sizes. Lying time, standing time, and bouts were recorded using accelerometers (Onset HOBO Pendant G) at 1 min intervals for 14 d. Statistical analyses were performed using the GLIMMIX and MIXED procedures of SAS (v9.4). Heifers receiving SPRAY had lower number of licks received per day and lower tail paint removed regardless of day or follicle size when compared to CON or TRTA. Rump lick received, chin rest received, anogenital sniff received, mount received, and both initiated and

received behaviors for attempt to mount occurred more in heifers with large follicles on d 8 and d 10. Heifers spent more time standing and less time lying per day when they had a large follicle compared with a small follicle. Dairy operations that have problems with tail paint removal and false-positives may benefit from changing to a tail paint product with a different consistency, such as a spray formulation. Producers looking for heifers to breed should focus on those receiving rump lick, chin resting, anogenital sniff, mount, and attempt to mount, or increases in daily activity. The use and combination of these heat detection tools will improve reproductive efficiency and save resources.

Key Words: Tail paint, behavior, heifers

INTRODUCTION

The use of tail paint as an estrus detection aid dates back to Victorian and New Zealand dairy farms in the late 1970's (Macmillan and Curnow, 1977). The paint strip method detects cows that are in estrus by indicating those which have been mounted, resulting in the tail paint being rubbed off. Using this estrus detection aid and visual observation, New Zealand herds achieved an AI rate > 90% (Macmillan and Curnow, 1977). Moreover, the tail paint method was able to correctly identify 6% of the New Zealand herd that was not detected by owners (Macmillan and Curnow, 1977). Estrus detection efficiencies using a tail paint method have been reported to be > 98% in dairy cows (Macmillan and Curnow, 1977; Xu et al., 1998) and > 94% in heifers (Macmillan et al., 1988). One difficulty to the tail paint system is the possibility of false-positives, when cows are detected by the tail paint to be in estrus but are not (Pennington and Callahan 1986). Tail paint has been shown to result in 5% false positives (Macmillan and Curnow, 1977) and therefore causes producers to doubt the efficacy for detecting estrus. Previous studies have involved enamel paint, tail chalk, and a combination of tail paint plus raddle marking (Macmillan and Curnow, 1977; Pennington and Callahan, 1986; and Macmillan et al., 1988). However, literature is scarce on studies comparing multiple tail paint formulations.

Like dairy cows, heifers are no exception to reproductive problems and can also contribute to economic losses from delay in getting pregnant due to poor estrus detection. In a nationwide survey, US dairy producers identified inadequate estrus detection and lack of time to supervise the animals as 2 important reasons for not using AI to breed dairy heifers (Erven and Arbaugh, 1987). It has been estimated that the US dairy industry loses approximately $300 million yearly to erroneous diagnosis and failure to detect estrus (Senger, 1994). Although heifers usually have better conception rates than cows, with a mean rate of 57% in 2005

(Kuhn et al., 2006), failure in estrus detection and consequently, in breeding those animals, may lead to poor reproductive efficiency.

Early studies have reported success in using pedometers (Farris, 1954; Kiddy, 1976) and accelerometers (Ito et al., 2009; Ledgerwood et al., 2010; Løvendahl and Chagunda, 2010) for estrus detection. Using both types of activity monitors, literature has shown a definite increase in activity at the time of estrus (Farris, 1954; Hurnik et al., 1975) with animals in estrus spending more time standing and less time lying. Kiddy (1976) reported an increase in activity at the time of estrus in 93% of estrous periods and heat detection from a pedometer for 21% of cows in free stalls that were not detected by herdsmen.

Behavioral studies have mainly focused on lactating dairy cow behavior, and most of the studies focusing on estrus behaviors in dairy heifers were done over two decades ago (Esslemont et al., 1980; Helmer and Britt, 1985; Kerbrat and Disenhaus, 2004). Traditionally, standing estrus has been defined as the period in which a cow makes no effort to escape when being mounted (Hurnik et al., 1975). Thus, standing for mounting has been the primary sign of true estrus, but has been reported in very low frequencies in literature and can be easily overlooked (Kerbrat and Disenhaus, 2004; Sveberg et al., 2011). In addition, it has been reported that some cycling animals have "silent heats" in which mounting behavior is not performed (Foote, 1975). Therefore, observing other signs associated with estrus have generated higher heat detection rates (Van Eerdenburg et al., 1996). Early studies did not regard chin resting as a sign of estrus (Foote, 1975), but later research declared chin resting as a positive indicator of heat along with vulva sniffing and aggressive behaviors (Hurnik et al., 1975; Esslemont et al., 1980; Sveberg et al., 2011). To aid the understanding of behaviors in dairy cattle, classifications have been made such as: estrus interactions: those which are associated with standing estrus in literature; agnostic

interactions: those that are aggressive or threatening to others; and social interactions: those that occur when an animal shows interest in another without any threatening, aggressive, or submission postures (Kerbrat and Disenhaus, 2004). Social interactions (such as licking or rubbing behaviors) may lead to the removal of tail paint and consequently result in false-positives for estrus detection. Therefore, the main objective of this study was to compare the behaviors associated with 3 different types of tail paint formulations in of Holstein heifers.

MATERIALS AND METHODS

Animals and Housing

The University of Illinois Institutional Animal Care and Use Committee approved all following experimental procedures. Eighteen (n = 18) Holstein heifers were balanced according to their age (13.7 ± 1.2 mo), body weight (**BW**; 394 ± 32 kg), and body condition score (**BCS**; 3.43 ± 0.1, on a scale of 1 = emaciated to 5 = obese) and housed in free stalls with sand bedding and headlocks at the University of Illinois Dairy Cattle Research Unit (Champaign-Urbana, Illinois). All heifers received the same total-mixed ration fed once daily (~ 1200 h) to fulfill the requirements outlined by NRC (2001). The experimental period was 6 wk between August 8 and September 4, 2013.

Experimental Design and Treatments

The experiment was performed using a 3×3 replicated Latin Square design with 3 animals per square and 6 total squares for 3 periods of 14 d each. The heifers were randomly assigned to one of 3 treatments in each period: control (**CON**), a commercially available chalk formulation; treatment-A (**TRTA**), a chalk formulation with an added proprietary ingredient designed to discourage licking of the chalk; or treatment-B (**SPRAY**), a commercially available

formulation with the same ingredients as CON but with a spray paint consistency. All treatments were orange in color (All Weather PaintStik, LA-CO Industries, Elk Grove, IL). Treatments were refreshed once daily before feeding time. Old treatments were completely removed at the end of each period prior to application of the new treatment. Treatments were evaluated once daily before re-application to score the degree of tail paint removed (**TPR**). If no paint was removed from the previous day, the score was 0; if less than half was removed, the score was 1; and a score of 2 was given if more than half or all was removed (Figure 2.1).

Estrus Synchronization and Follicle Size

An Ovsynch protocol was used starting on d 1 of each period (d 1: GnRH: 2mL of Factrel, Zoetis, Florham, NJ); d 8: $PGF_{2\alpha}$: 5 mL of Lutalyse, (Pfizer Animal Health, New York City, NY); d 10: GnRH to stimulate periods of high and low interactions. The protocol was not used for timed AI, but only as an attempt to stimulate groups of heifers to demonstrate estrus together for better detection of estrus. All injections were given intra-muscularly in the rear leg. Ovaries were examined via ultrasound imaging using the Ibex Pro portable ultrasound (E.I. Medical Imaging, Loveland, CO) with L6.2 transducer (8-5MHz 66-mm linear array, 12 cm scan depth) on d 1, 8, and 10 of each period. The transducer was inserted into the rectum and placed over the broad ligament and uterine horns to examine the ovaries. Both the right and the left ovaries were examined and images were captured to determine if structures were present. The presence of follicles or corpus luteum was recorded and Image J (U.S. National Institutes of Health, Bethesda, MD) was used to measure follicle size. All follicles were measured using an image with a known length (mm), measuring the pixels of the known length, and calibrating the scale from pixels to mm. Hormone injections and ultrasound were done prior to daily feeding.

Activity

Lying time and standing time were recorded using electronic data loggers (Onset HOBO Pendant G Acceleration Data logger, Onset Computer Corporation, Pocasset, MA) at 1-min intervals for 14 d. The accelerometers were attached with vet wrap (3M Vetrap Bandaging Tape, Neuss, Germany) and placed horizontally on the outside of the left hind leg. The loggers recorded the *g*-force on the x, y, and z-axes and was situated such that the x-axis was parallel to the ground, the y-axis was perpendicular to the ground pointing upward, and the z-axis was parallel to the ground pointing toward the sagittal plane. Accelerometers were collected on d 14 of each period and a new logger was put on at the same time. Accelerometers were read using HOBOware software (Onset Computer Corporation), which converted *g*-force readings into degrees of tilt. The data was exported into Microsoft Excel files and the degree of vertical tilt (y-axis) was used to determine the lying position of the heifers. A macro was used in SAS (v9.4; SAS Institute Inc., Cary, NC) to calculate daily standing time and lying time (min/d) based on 1,440 observations from midnight to midnight the following day (Ito et al., 2009). Standing and lying time, standing and lying duration, and bouts were analyzed.

Behavior Observation

Each day, 30-min observations were performed every 2 h from 6 am to 6 pm, for a total of 7 time-points per day. A total of 13 behaviors were observed, adapted from Sveberg et al. (2011). The following behaviors were not observed during this trial: avoid, threat, chase away, flehmen, bellow, follow, lean head, side mount, and head mount. Notes were taken to identify which heifer was the initiator or the receiver, with the exception of play rub, where the initiator and receiver could not be clearly distinguished. Definitions of all behaviors can be seen in Table 2.1. In attempt to give a more clear definition, we modified the following behaviors from

Sveberg et al. (2011): Winner: The initiator wins in an agnostic interaction over a resource (such as feed or water) or an interaction in which the behavior cannot be defined, and the receiver (the loser) moves away or changes position. In addition, we included the following behavior and definition to fit the objectives of the study: paint lick (the initiator consistently licks the tail paint of the receiver). Videos were watched retrospectively to verify the observations and record any missed behaviors. One person did this to ensure accuracy. The behaviors were entered into Microsoft Excel as counts of occurrences.

Statistical Analyses

All statistical analyses were performed using SAS (v9.4; SAS Institute Inc., Cary, NC). Behavior counts were summed for each 30-minute time-point with 7 variables per day and TPR had just one variable per day: the score for the degree of product removal. For all analyses, the experimental unit was heifer. The frequencies of traits for all observation time-points in 3 periods were analyzed using PROC FREQ and graphs for 4 behaviors related directly to identifying how heifers respond to the tail paint treatments were generated (Figure 2.2). The following behaviors were considered related to the treatments: paint lick, social lick, rump lick, and anogenital sniff. Paint lick was selected because it directly related to licking behavior and TPR. The other behaviors were selected because they may have been mistaken for paint lick or could have demonstrated heifers showing interest in the treatments. In addition, the frequency graphs shown were only for the received behaviors because the treatments on the receiving heifer were affected.

Behaviors were analyzed with a Poisson distribution in PROC GLIMMIX. The model contained heifer as a random effect and the fixed effects of period, treatment (when applicable), and week. Least squares means were calculated for tail paint treatments of related behaviors and

a Tukey's adjustment was used for controlling multiple comparisons error rate. The incidence rate ratio was also determined for the aforementioned behaviors. The incident rate ratio represents the change in the first treatment when compared to the second treatment in terms of a percentage increase or decrease; with the percentage determined by the amount the rate ratio was above or below 1. The PROC MEANS procedure was used to demonstrate the mean frequency per week of the behaviors and TPR, averaged by all observation time-points in each week. Least squares means were also calculated for traits by comparing the 2 wk of each period. Heifers were expected to come into estrus during the second week from the synchronization protocol, therefore, d 1 to 7 was considered a time of low activity and d 8 to 14 was considered a time of high activity.

The measurements for all follicles were ranked in order from smallest to largest in size. This list was then broken into terciles to determine cut-off values for a small, medium, or large follicle. Since estrus was expected in the second week, the follicular data from d 8 and d 10 were compared to the behaviors and TPR. The counts of occurrences for each behavior on d 7, 8, and 9 were summed together and compared to the follicular data from d 8. For the follicular data on d 10, the behavior counts for d 10, 11, and 12 were summed together. The summations of behavior counts were done in order to better detect a difference in estimates. However, standing and lying data was not summed because there were more observations in one day versus the behavior observations. Follicular data, standing activity, and lying activity, were analyzed using the PROC MIXED procedure with heifer as a random effect and the fixed effects of period, follicle size, treatment (when applicable), and day of ultrasound. Day of ultrasound was analyzed as a repeated measure. Statistical significant declared as P value lower than 0.05, and tendency

declared as P value lower than 0.10. A tendency was declared for the treatment × time interaction when P value was lower than 0.10.

RESULTS

Least squares means of treatments for TPR and related behaviors can be seen in Table 2.2. Paint lick received, anogenital sniff received, and TPR had significant treatment differences ($P < 0.001$, $P = 0.04$, and $P < 0.001$, respectively). SPRAY had a lower treatment mean for TPR and paint lick received (1.80 and 1.18, respectively) than either CON (6.78 and 2.50) or TRTA (5.65 and 2.33). However, SPRAY received more anogenital sniffs than CON, and TRTA was not different from either CON or SPRAY. The treatment by wk interaction was not significant for any trait ($P > 0.29$).

The Poisson regression model for the related traits with significant treatment differences (Table 2.3). The CON treatment was 272% more likely to be removed compared with SPRAY ($P < 0.001$). A tendency was observed for CON to be removed 20% more than TRTA ($P = 0.06$), and SPRAY was 68% less likely to be removed than TRTA ($P < 0.001$). No significant difference was detected ($P = 0.63$) in paint lick received between the two chalk formulations (CON and TRTA), with CON being slightly (7%) more likely to be licked than TRTA. In addition, CON was 112% more likely to be licked than SPRAY ($P < 0.001$) and SPRAY was 49% less likely to be licked than TRTA ($P < 0.001$).The opposite was observed for anogenital sniff. The CON treatment was 27% less likely to receive an anogenital sniff than SPRAY ($P = 0.01$).

Analysis of expected low activity and high activity is reported in Table 2.4. A significant difference in wk was observed for tail paint removed ($P < 0.01$) and for both initiated and

received behaviors for: paint lick ($P = 0.05$), social lick ($P = 0.01$), rump lick ($P = 0.05$), body butt ($P < 0.01$), chin rest ($P < 0.01$), and mount ($P < 0.01$). Of these, the behaviors that may be more related to social interactions (paint lick, social lick, and rump lick) were observed more frequently in wk 1 when heifers were expected to exhibit low activity. Conversely, the estrus and agnostic behaviors (body butt, chin rest, and mount) were more frequently observed in wk 2 when heifers were expected to come into heat and have higher activity.

Cows that received TPR also resulted in a significant increase ($P < 0.01$) for wk 1, however this was opposite of what we expected. Figures 2.4 and 2.5 show results to further investigate this significance. Figure 2.4 illustrates an interaction between treatment and follicle size. The treatment by follicle size had a tendency ($P = 0.09$) and there was a significant treatment effect ($P < 0.001$) as expected. Heifers with small follicles receiving CON and TRTA had less TPR than those receiving SPRAY ($P < 0.001$) and heifers with large follicles receiving CON had more TPR than those receiving SPRAY ($P < 0.02$). In addition, Figure 2.5 shows an interaction between treatment and day of ultrasound. Heifers had more TPR for CON versus SPRAY on d 8 and d 10 ($P < 0.02$). Heifers had more TPR for TRTA versus SPRAY on d 8 ($P < 0.001$) and a tendency on d 10 ($P < 0.08$).

Using follicular measurements, a large follicle was determined to be 12.4 mm or greater (Table 2.5). On d 8, there were 10 heifers with a large follicle in the first period, 7 in the second period, and 6 in the third period. On d 10, there were 5 heifers with a large follicle in the first period, 9 in the second period, and 10 in the third period. We considered heifers that had a large follicle in the absence of a CL to be in estrus. We observed differences for rump lick received ($P < 0.01$), chin rest received ($P < 0.01$), anogenital sniff received ($P = 0.02$), mount received ($P < 0.01$), and both initiated and received behaviors for attempt to mount ($P = 0.03$ and $P < 0.01$). A

tendency was observed for differences in mount initiated *(P* = 0.08) and both winner initiated and winner received (*P* = 0.07). For all traits with a significant difference in follicle size, the behaviors occurred more often on d 8 and on d 10 when there was a large follicle, compared with heifers without a large follicle. The following behaviors had significant day effects and occurred more often on d 10, versus d 8, for heifers with and without a large follicle: mount received (*P* = 0.03), anogenital sniff initiated (*P* = 0.04), anogenital sniff received (*P* = 0.02), chase up initiated (*P* = 0.03), and chase up received (*P* = 0.02). Conversely, push initiated and received (*P* = 0.04) had a significant day effect, but occurred more on d 8 for heifers with and without a large follicle. Winner initiated also increased when heifers had a large follicle (*P* = 0.07). Follicle size and day interaction was also considered, but was not significant for any trait (*P* > 0.13), with the exception of winner initiated (*P* > 0.06).

Heifers spent more time standing (*P* < 0.01) and less time lying (*P* < 0.01) when they had a large follicle (Table 2.6). Likewise, heifers spent more total daily time standing and lying (P < 0.001) and had longer durations standing and lying (*P* = 0.001) on d 10, when there were more heifers with a large follicle. Follicle size and day interaction was also considered, but was not significant for any trait (*P* > 0.20).

DISCUSSION

Social lick and anogenital sniff were the most frequently observed behaviors when compared with paint lick and rump lick (Figure 2.2). Approximately 25% of all observations resulted in heifers never receiving a social lick, whereas we observed heifers never receiving paint lick or rump lick 76% and 90% of the time, respectively. Anogenital sniff occurred the most at any one time with up to 13 observations in a 30-minute period. However, social lick was the only where heifers received at least one social lick for more than 50% of all observations.

Although paint licking is reported on dairy farms, the behavior is very rare compared to other types of licking. Heifers received more than one paint lick less than 2% of all observations. These behaviors can also be seen relative to all the other behaviors and TPR in mean counts for 30-minute observations in Figure 2.3. Of all behaviors, initiated and received for mount, chase up, and rump lick had the lowest incidence and social lick initiated and received had the highest.

Anogenital sniff has been shown to occur in high frequency and to increase at times of estrus in both heifers and cows (Esslemont and Bryant, 1976; Kerbrat and Disenhaus, 2004; Sveberg et al., 2011). Results from the present study agree with previously reported data, showing anogenital sniff as the second most frequently observed behavior on average, after social lick, and a significant increase in anogenital sniff received when heifers have large follicles. The increase in anogenital sniff received when heifers had large follicles is possibly from vaginal secretions that occur when heifers are in estrus. Literature has also shown that anogenital sniffs are sometimes performed in non-estrus states (Phillips and Schofield, 1990; Van Eerdenburg et al., 1996). We also observed anogenital sniff being initiated and received in heifers that did not have large follicles and reason that this behavior alone should not be used to determine which heifers to breed.

A low frequency of rump lick received in the present study also agrees with low counts reported in literature (Sveberg et al., 2011). Conversely, the same authors reported a low frequency of social licking (< 0.2 counts per hour) and our results show that social licking in heifers was observed slightly more, with a mean of > 0.2 counts in a 30-min period. This may be from a difference in heifers used in the present trial, compared with cows used by the authors. Heifers are younger, weigh less, and are non-lactating, which may drive them to be more active and perform more behaviors.

Producers have concerns that heifers may lick the tail paint and yield false positives. We observed a low frequency of tail paint being licked by heifers when compared to the other licking behaviors. It is possible that licking seen on commercial dairy farms may be primarily from social licking rather than heifers licking the tail paint. Paint licking and rump licking may also be mistaken for anogenital sniffing, a more frequent behavior.

Heifers with the SPRAY treatment received more anogenital sniffs. This could indicate that heifers show interest in that particular tail paint treatment compared with the others. However, heifers with SPRAY received fewer paint licks and had lower TPR compared to CON and TRTA. This may be from the different consistency of the SPRAY treatment (spray paint) versus the chalk formulations of CON and TRTA. No differences were found in paint lick or anogenital sniff received between CON and TRTA. The experimental ingredient in TRTA did not seem to deter the interest of the heifers compared with CON since there was no difference in licks or sniffs, however TRTA tended to be removed less and may yield less false positives.

During wk 1, when we expected activity to be low, there was more product removed (greater TPR). This is opposite of what we expected: greater TPR with increased mounting in wk 2, since we expected more heifers in het during wk 2 and mounting should cause product removal. These results may be from the differences in treatments. Treatments CON and TRTA were removed more than SPRAY regardless of the follicle size or day, with the exception of heifers with large follicles receiving TRTA (Figures 2.4 and 2.5). This could also have been from the increase in social behaviors during wk 1 or from heifers with large follicles that were not noticed since ultrasound was only performed on the first day for wk 1.

Agnostic and estrus behaviors that occurred more during high activity in wk 2 include body butt, chin rest, and mount. Both initiated and received behaviors were increased and this

was expected since heifers should have been in estrus during wk 2. This agrees with previous studies that showed higher incidence of agnostic behaviors in cows during times of estrus (Hurnik et al,. 1975; Sveberg et al., 2011). However, the decrease in social licking during wk 2 disagrees with results from Sveberg et al. (2011) that showed higher incidence of social licking during times of estrus. This difference may be explained by the much higher frequency of social licking in the present study compared to that of Sveberg et al. (2011).

Results from this study have shown a higher incidence for received estrus behaviors (chin rest, anogenital sniff, mount, and attempt to mount) in heifers with large follicles versus heifers without large follicles on both d 8 and d 10 (Table 2.5). Mounting is still considered the gold standard for estrus detection, however it can easily be missed from lack of observation times, short duration of the behavior, or because some animals just do not show signs of mounting when in estrus (Hurnik et al., 1975; Sveberg et al., 2011). Van Vliet and Van Eerdenburg (1996) reported that just 37% of estruses were accompanied by standing mounts, and Kerbrat and Disenhaus (2004) noted that mounting only represented 8% of all estrus behaviors. Producers should look to the other agnostic behaviors to determine breeding prospects. However, caution should be taken if only chin resting and anogenital sniffing received is observed since these behaviors can be performed in nonestrus stages and are less predictive than mounting (Sveberg et al., 2011; Phillips and Schofield, 1990). Rump lick received also had a higher incidence in heifers with a large follicle and may be combined with the received agnostic behaviors to identify heats.

Looking to the initiated behaviors, both mount and attempt to mount had higher incidences in heifers with large follicles. This agrees with previous research that heifers in estrus attempt more mounts than in other estrous stages, followed by pro-estrus heifers attempting more

mounts than non-estrus heifers (Helmer and Britt, 1985). Furthermore, Hurnik et al. (1975) reported that 79% of all attempted mounts were performed by animals in estrus and that 90% of mounted animals were in estrus. We can reason that if mounting is observed in heifers, both the initiator and receiver may be in estrus or close to estrus. Unlike previous studies with cows, our results did not indicate differences for heifers with or without large follicles for head butt or chase up received (Hurnik et al., 1975; Sveberg et al., 2011).

Higher incidences of initiated and received estrus behaviors like chin rest, anogenital sniff, and mount occurred on d 10 versus d 8. This increase is most likely from more heifers having large follicles in two out of three periods on d 10. This finding is supported by studies that have shown a proportional increase in mounting frequency when there is a simultaneous increase in the number of animals in estrus (Hurnik et al., 1975; Esslemont et al., 1980; Helmer and Britt, 1985). We also saw a decrease in push initiated and received on d 8, when less heifers had large follicles, which agrees with findings from Sveberg et al. (2011) that reported a decrease in push initiated during times of estrus. The lower incidence of this behavior may be from the few heifers on d 8 that were coming into heat. Hurnik et al. (1975) described how a cow alone in estrus will nudge (or push) other cows in an effort to arouse them. It would follow that push decreases on d 10 because more heifers have large follicles on d 10 and are participating in estrus behaviors with each other. Thus, heifers with large follicles on d 10 have more partners to interact and do not need to seek out other heifers, versus few heifers that have large follicles (such as d 8) and will push other heifers more in order to arouse them. There were also significant effects for winner initiated for follicle size and the interaction of follicle size by day, however, it is unclear why this occurred.

Heifers with large follicles spent more time standing and less time lying each day. When there was an increased number of heifers with large follicles, our results showed increased total daily standing time and increased bout (how long heifers stand at one time). Likewise, heifers with large follicles spent less total time lying each day and less time lying before standing again than heifers with small follicles. The results from an accelerometer in the present study supports findings of increased activity at the time of estrus using pedometers and video surveillance in previous studies (Hurnik et al., 1975; Kiddy, 1976; Kerbrat and Disenhaus, 2004). Activity monitors have been shown to catch increased activity in 93% of estrus periods and detect 21% of estruses missed by herdsmen (Kiddy, 1976). The use of pedometers or accelerometers can be a reliable aid to heat detection on dairies for both heifers and cows.

CONCLUSIONS

Dairy operations that have problems with tail paint removal and false-positives may benefit from changing to a tail paint product with a different consistency, such as a spray formulation. Producers observing behaviors for heat detection can focus on heifers receiving rump lick, chin resting, anogenital sniff, mount, and attempt to mount. Caution must be used when observing licking, chin resting and anogenital sniff since they can also be performed in non-estrus stages. Likewise, heifers that initiate mounts, attempt to mount, or push/nudge other heifers should also be considered for breeding and may be estrual or pre-estrual. Lastly, producers can make use of activity monitors and should focus breeding efforts on heifers that have increased standing times. The use and combination of these heat detection techniques may improve reproductive efficiency in dairy operations.

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TABLES AND FIGURES

Table 2.1. Behaviors Observed

Classification	Behavior	Explanation
Social interactions	Paint lick	Initiator licks the tail paint of the receiver
	Social lick	Initiator licks the head, flank, or neck of the receiver
	Rump lick	Initiator licks the rump region of the receiver
	Play rub	Rubs head against another cow, except chin resting[1]
Agnostic interactions	Body butt	Initiator pushes forehead against part of body of the receiver, other than the head
	Head butt	Initiator bows head and pushes forehead firmly against forehead of the receiver
	Push	Initiator pushes the receiver with her head, resulting in the receiver moving away or changing position
	Winner	Initiator wins in an agnostic interaction over feed or resources or an interaction in which the behavior cannot be defined, where the receiver moves away or changes position
	Chase up	Initiator touches or butts a lying receiver, and the receiver stands up
Estrus interactions	Chin rest	Initiator rests her chin on the rump of the receiver
	Anogenital sniff	Initiator licks or sniffs the anogenital region of the receiver
	Mount	Initiator mounts and succeeds in resting both legs on rump with or without a standing response from the receiver
	Attempt to mount	Initiator tries to mount the receiver by raising front limbs, but does not succeed

[1] Behavior in which an initiator and receiver could not be clearly defined.

Table 2.2. Least squares means and associated standard errors of the mean (SEM) for the degree of tail paint removal (TPR) and behaviors related to the tail paint treatments for 18 dairy heifers

Trait	Treatment[1] Means			SEM	*P*-value[4]
	CON	SPRAY	TRTA		
TPR[3]	6.78[a]	1.8[b]	5.65[a]	0.25	<0.001
Behaviors initiated					
Paint lick	1.88[a]	1.84[a]	1.62[a]	0.35	0.77
Social lick	12.79[a]	13.70[a]	11.20[a]	1.44	0.25
Rump lick	1.08[a]	0.93[a]	0.71[a]	0.18	0.45
Anogenital sniff	5.46[a]	5.99[a]	4.01[a]	0.68	0.12
Behaviors received					
Paint lick	2.50[a]	1.18[b]	2.33[a]	0.18	<0.001
Social lick	13.54[a]	13.30[a]	12.40[a]	0.91	0.56
Rump lick	0.70[a]	0.95[a]	0.94[a]	0.17	0.59
Anogenital sniff	4.34[b]	5.96[a]	4.91[a,b]	0.51	0.04

[1]CON: control. SPRAY: spray formulation. TRTA: treatment-A, test product.
[3]TPR score: 0= no paint removed; 1= less than half of the paint removed; 2= more than half of the paint was removed. Scores for dairy heifers were summed by week.
[4]Treatment by week interaction was not significant for any trait ($P > 0.29$).
[a,b]Values with the same letter are not significantly different as determined by a Tukey's adjustment.

Table 2.3. Incident Ratio from Poisson regression for traits with significant treatment differences by comparison of tail paint treatments for 18 dairy heifers

Trait	Coefficient	SE[1]	IRR[2]	95% CI[3]	*P*-Value[4]
Tail paint removed (TPR)[5]					
CON-SPRAY[6]	1.315	0.14	3.72	2.83 - 4.90	<0.001
CON-TRTA	0.180	0.09	1.20	0.99 - 1.45	0.06
SPRAY-TRTA	-1.135	0.14	0.32	0.24 - 2.34	<0.001
Paint lick received					
CON-SPRAY	0.750	0.18	2.12	1.49 - 3.06	<0.001
CON-TRTA	0.071	0.15	1.07	0.80 - 1.43	0.63
SPRAY-TRTA	-0.679	0.18	0.51	0.35 - 1.37	<0.01
Anogenital sniff received					
CON-SPRAY	-0.316	0.12	0.73	0.57 - 2.03	0.01
CON-TRTA	-0.123	0.13	0.88	0.69 - 1.14	0.34
SPRAY-TRTA	0.193	0.12	1.21	0.96 - 1.54	0.11

[1]SE= standard error.
[2]IRR: incidence rate ratio.
[3]CI= confidence interval.
[4]Treatment by week interaction was not significant for any trait ($P > 0.29$).
[5]TPR Score: 0= no paint removed; 1= less than half of the paint removed; 2= more than half of the paint was removed. Scores for dairy heifers were summed by week.
[6]CON: control. SPRAY: spray formulation. TRTA: treatment-A, test product.

Table 2.4. Least squares means values of recorded behaviors and degree of tail paint removed (TPR) by comparison of expected low activity in week 1 and expected high activity in week 2

Trait	Week 1- low activity[1]		Week 2- high activity		
	Mean	95% CI[2]	Mean	95% CI	*P*-Value
TPR[3]	5.82	4.89 - 6.92	4.01	3.28 - 4.91	<0.01
Play rub	1.67	1.24 - 2.25	1.55	1.15 - 2.10	0.64
Behaviors initiated					
Paint lick	2.11	1.46 - 3.03	1.50	1.02 - 2.21	0.05
Social lick	14.35	11.36 - 18.12	11.06	8.68 - 14.08	0.01
Rump lick	1.19	0.85 - 1.66	0.71	0.47 - 1.09	0.05
Body butt	2.34	1.76 - 3.12	3.46	2.64 - 4.55	<0.001
Head butt	3.68	2.84 - 4.76	4.15	3.22 - 5.35	0.29
Push	4.01	2.95 - 5.44	3.88	2.85 - 5.27	0.77
Winner	4.22	2.86 - 6.21	4.23	2.87 - 6.24	0.97
Chase up	0.41	0.24 – 0.72	0.61	0.37 – 1.01	0.20
Chin rest	1.88	1.23 - 2.87	3.72	2.63 - 5.27	<0.01
Anogenital sniff	4.89	3.72 - 6.41	5.58	4.30 - 7.24	0.41
Mount	0.35	0.17 - 0.75	1.20	0.73 - 1.95	<0.01
Attempt to mount	0.60	0.34 - 1.09	0.66	0.38 - 1.17	0.77
Behaviors received					
Paint lick	2.42	2.00 - 2.93	1.66	1.33 - 2.09	0.01
Social lick	14.96	13.21 - 16.93	11.50	10.07 - 13.14	<0.001
Rump lick	1.16	0.86 - 1.57	0.66	0.44 - 0.99	0.03
Body butt	2.21	1.57 - 3.11	3.29	2.37 - 4.57	<0.01
Head butt	3.67	2.81 - 4.78	4.09	3.14 - 5.32	0.29
Push	4.12	3.16 - 5.38	4.02	3.08 - 5.25	0.82
Winner (loser)	4.93	4.04 - 6.02	4.91	4.02 - 6.00	0.97
Chase up	0.33	0.17 – 0.64	0.50	0.27 – 0.94	0.11
Chin rest	1.88	1.25 - 2.84	3.78	2.71 - 5.28	<0.01
Anogenital sniff	4.74	3.85 - 5.85	5.48	4.48 - 6.71	0.16
Mount	0.30	0.14 - 0.62	1.09	0.66 - 1.78	<0.001
Attempt to mount	0.61	0.37 - 1.01	0.54	0.32 - 0.91	0.69

[1]Low activity is expected in week 1 of each period and high activity is expected in week 2 of each period due to the application of the Ovsynch protocol.
[2]CI= confidence interval.
[3]TPR Score: 0= no paint removed; 1= less than half of the paint removed; 2= more than half of the paint was removed. Scores for 18 dairy heifers were summed by week.

Table 2.5. Estimates of observed behaviors and degree of tail paint removed (TPR) by comparison of follicle size for 18 dairy heifers on day 8 and day 10 of 3 periods, as determined by ultrasound of ovarian structures

Trait	Day 8			Day 10			*P*-Value[3]	
	Follicle ≥12.4mm	Follicle <12.4mm	SE[1]	Follicle ≥12.4mm	Follicle <12.4mm	SE	Follicle Size	Day
TPR[2]	15.77	15.62	2.22	16.49	13.17	2.27	0.48	0.72
Play rub	0.83	0.54	0.16	0.67	0.56	0.17	0.27	0.69
Behaviors initiated								
Paint lick	0.68	0.72	0.20	1.03	0.44	0.21	0.20	0.86
Social lick	4.63	6.67	0.89	4.58	4.74	0.91	0.21	0.24
Rump lick	0.40	0.48	0.15	0.43	0.36	0.16	0.97	0.79
Body butt	1.88	1.80	0.32	1.71	1.37	0.33	0.52	0.33
Head butt	1.95	1.68	0.33	1.89	1.13	0.34	0.12	0.32
Push	2.48	2.13	0.38	1.76	1.46	0.39	0.36	0.04
Winner	2.03	2.05	0.39	2.73	1.58	0.40	0.07	0.67
Chase up	0.15	0.02	0.15	0.52	0.39	0.15	0.43	0.03
Chin rest	1.52	0.94	0.51	2.46	1.80	0.52	0.23	0.07
Anogenital sniff	2.25	1.78	0.49	3.69	2.55	0.51	0.14	0.04
Mount	0.29	0.17	0.23	1.26	0.49	0.27	0.08	0.008
Attempt to mount	0.84	0.21	0.19	0.51	0.26	0.20	0.03	0.45
Behaviors received								
Paint lick	0.93	0.66	0.15	0.65	0.75	0.15	0.61	0.56
Social lick	6.13	5.55	0.60	4.44	4.75	0.62	0.84	0.07
Rump lick	0.66	0.19	0.14	0.59	0.23	0.14	<0.01	0.92
Body butt	1.76	1.86	0.41	2.03	1.11	0.42	0.29	0.51
Head butt	1.91	1.71	0.33	1.54	1.40	0.33	0.61	0.27
Push	2.29	2.27	0.35	1.49	1.71	0.36	0.78	0.04
Winner (loser)	1.97	2.06	0.33	2.25	1.97	0.34	0.78	0.76
Chase up	0.20	0	0.14	0.35	0.52	0.14	0.86	0.02
Chin rest	1.70	0.838	0.55	3.36	1.08	0.56	<0.01	0.09
Anogenital sniff	2.24	1.79	0.47	4.18	2.15	0.48	0.02	0.02
Mount	0.47	0.04	0.21	1.22	0.36	0.22	<0.01	0.03
Attempt to mount	0.75	0.12	0.16	0.58	0.07	0.17	<0.01	0.53

[1]SE= standard error.
[2]TPR Score: 0= no paint removed; 1= less than half of the paint removed; 2= more than half of the paint was removed. Scores for dairy heifers were summed for each respective day, for all 3 periods of the trial.
[3]Follicle size by day interaction was not significant in for any trait ($P > 0.13$), with the exception of winner initiated ($P > 0.06$).

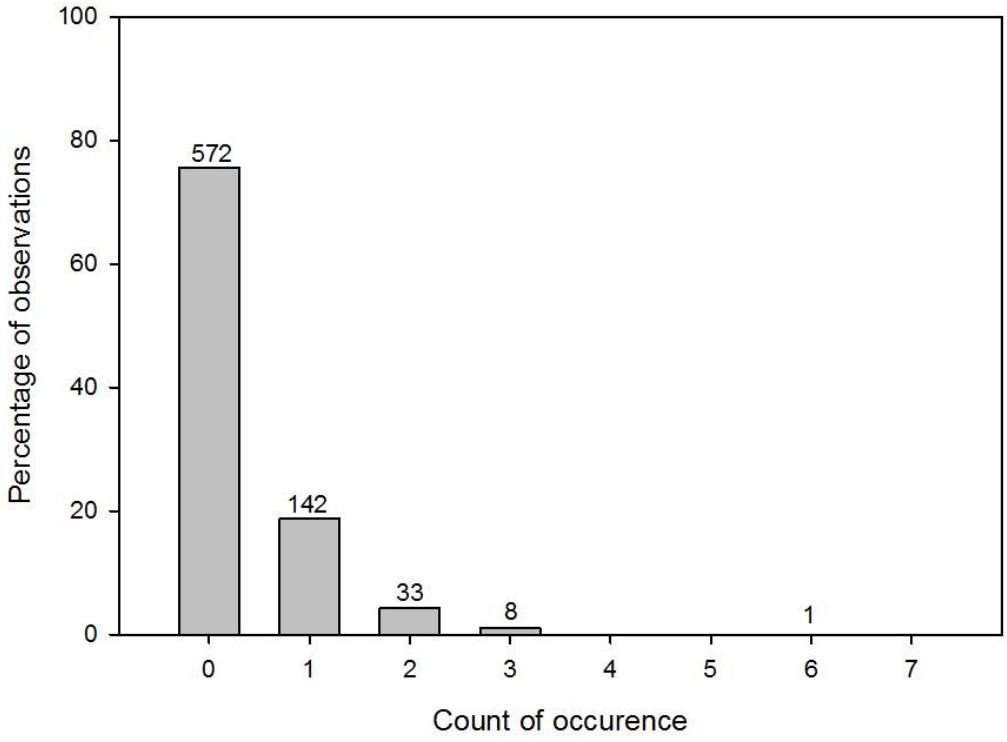
Table 2.6. Estimates of standing and lying behavior by comparison of follicle size for 18 dairy heifers on day 8 and day 10 of 3 periods, as determined by ultrasound of ovarian structures

Trait[1]	Day 8			Day 10			*P*-Value[2]	
	Follicle ≥12.4mm[2]	Follicle <12.4mm	SE[1]	Follicle ≥12.4mm	Follicle <12.4mm	SE	Follicle Size	Day
Standing time, total daily min	715.65	662.63	24.02	897.47	790.34	24.64	<0.01	<0.001
Standing duration, min	70.08	60.85	7.93	91.75	83.29	8.17	0.22	0.001
Standing bouts, n[3]	11.11	11.47	0.87	11.15	12.52	0.90	0.26	0.45
Lying time, total daily min	722.88	777.38	24.41	541.67	632.71	25.16	<0.01	<0.001
Lying duration, min	58.77	59.19	3.58	44.73	53.41	3.68	0.15	0.001
Lying bouts, n	13.62	13.83	1.18	12.29	14.39	1.22	0.31	0.72

[1]SE= standard error.
[2]Follicle size by day interaction was also considered but was not significant for any trait ($P > 0.20$).
[3]n=Number of bouts in 24 hours.

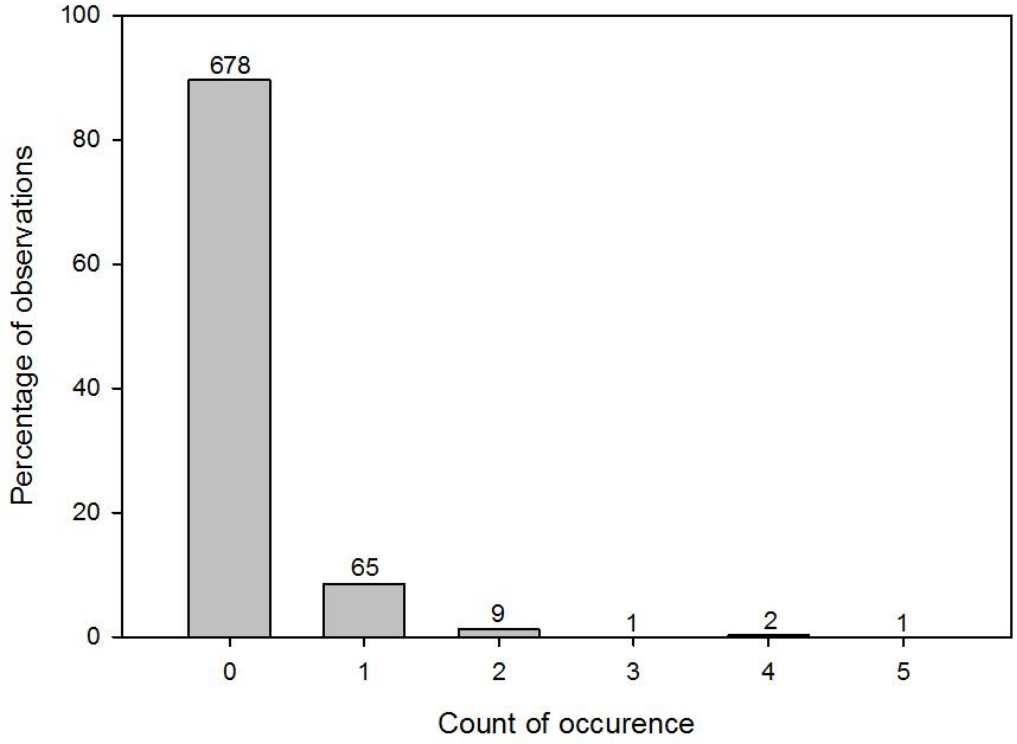
Figure 2.1. Tail paint removed (TPR) score.

A



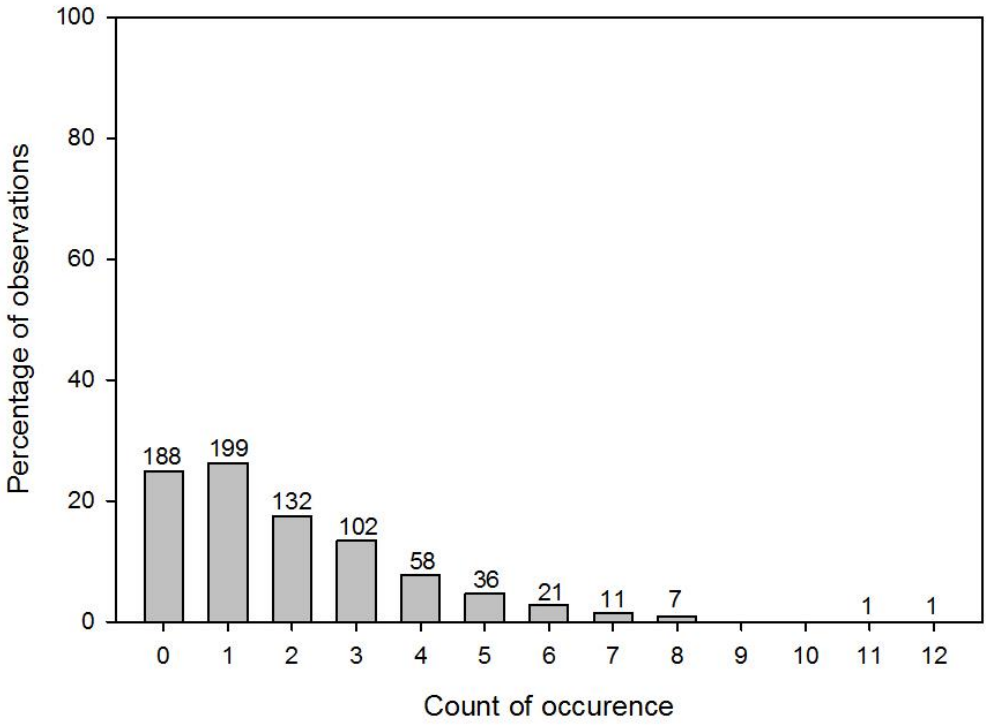
Skenandore/ Figure 2.2

B



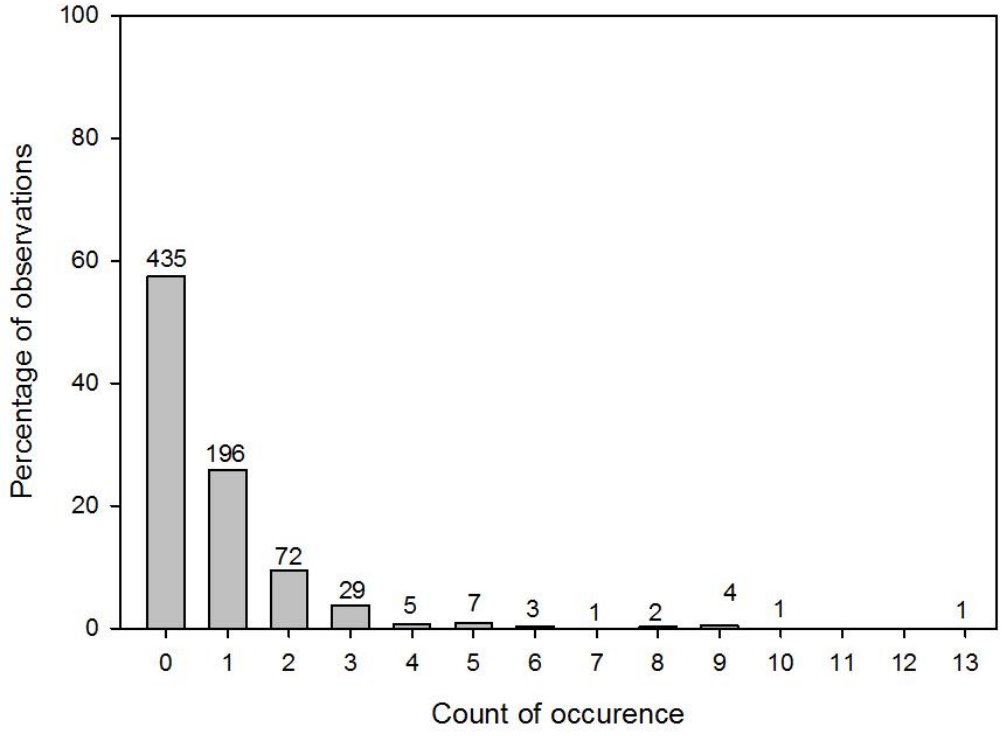
Skenandore/ Figure 2.2 (continued)

C



Skenandore/ Figure 2.2 (continued)

D



Skenandore/Figure 2.2 (continued)

Figure 2.2. Frequency of observations for behaviors related to the tail paint treatments. A: Paint lick received; B: Rump lick received; C: Social lick received; D: Anogenital sniff received. There were 756 total observations for all 18 heifers in the trial. The x-axis shows how many times in a single 30-min period the behavior was observed. The y-axis shows the percentage of the total observation each count makes up. The number of times that count was observed is shown directly above each bar.

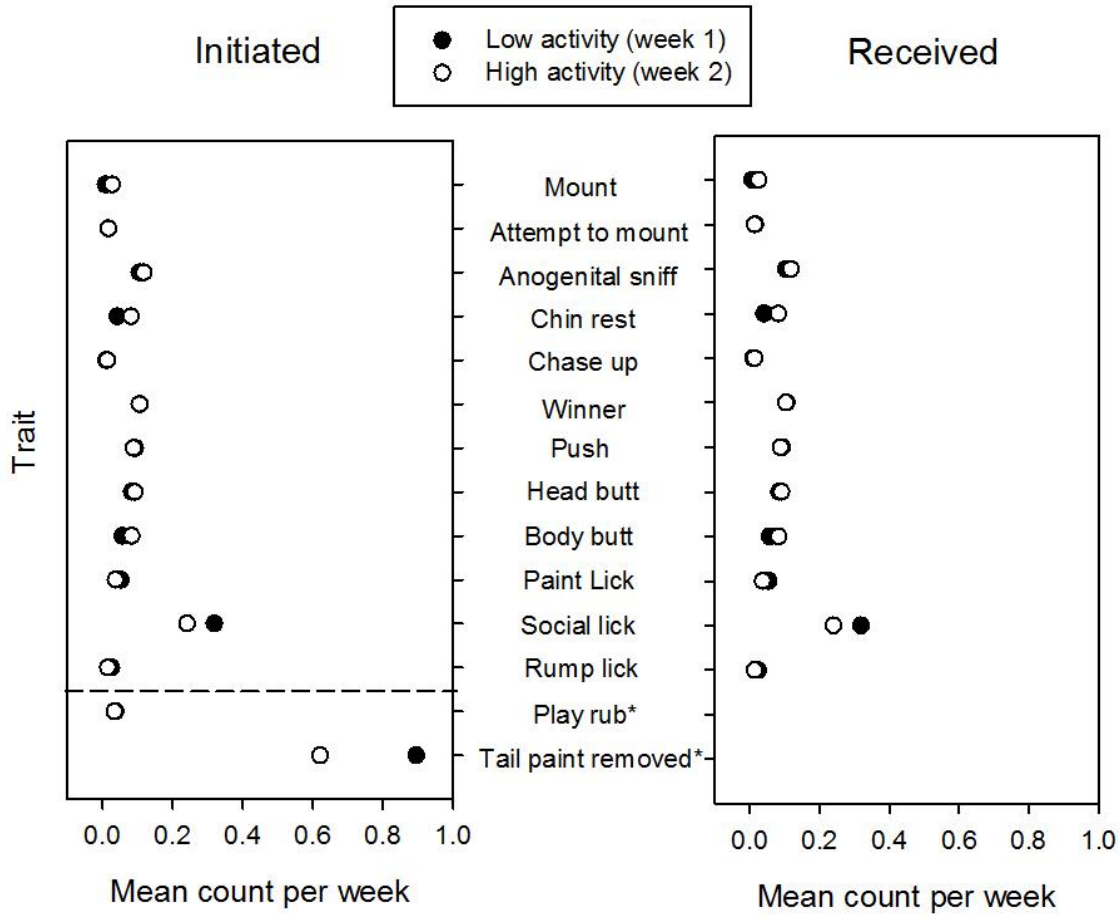


Figure 2.3. Mean counts of occurrences for all 30-minute observations of initiated and received behaviors and for degree of tail paint removed, for each week The left panel displays the behaviors initiated by heifers and traits in which an initiator and receiver could not be clearly defined (indicated with *) below the dashed line. The right panel displays the behaviors that were received by heifers.

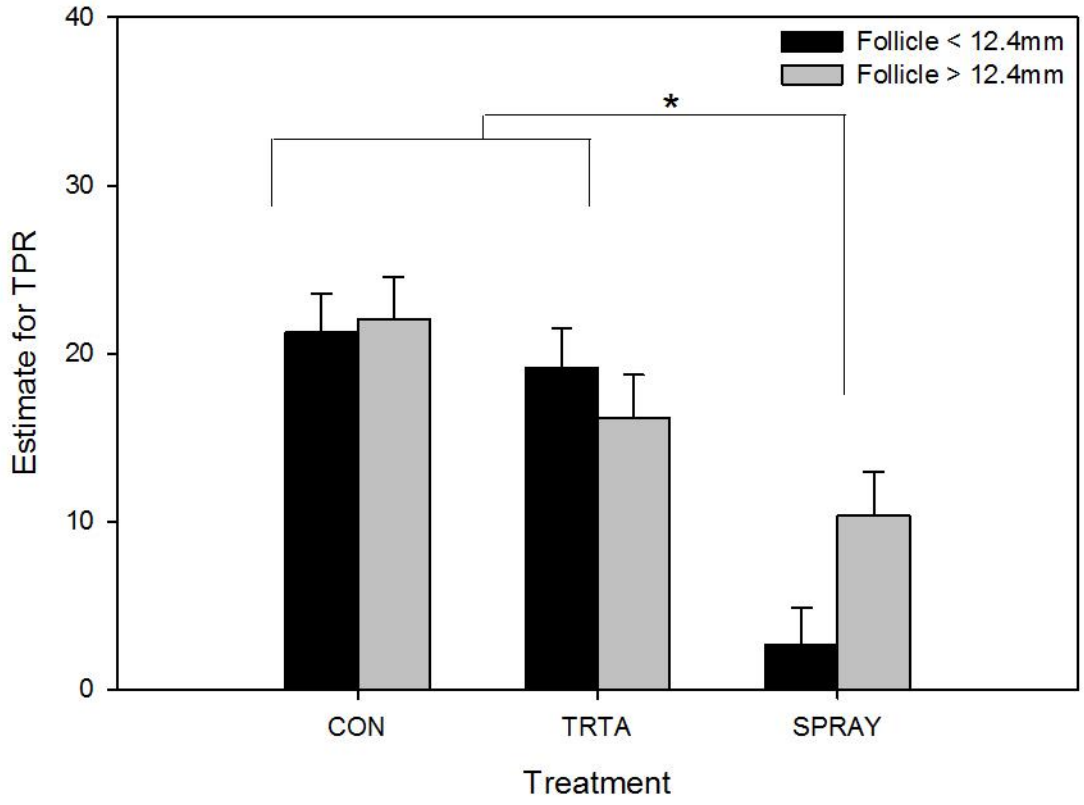


Figure 2.4. Estimate of tail paint removed (TPR) for effects of treatment, follicle size, and interactions. Treatment by follicle size: ($P = 0.09$); treatment: ($P < 0.001$). All other effects and interactions were not significant ($P < 0.20$). Estimates between follicle sizes differ significantly ($P < 0.02$).

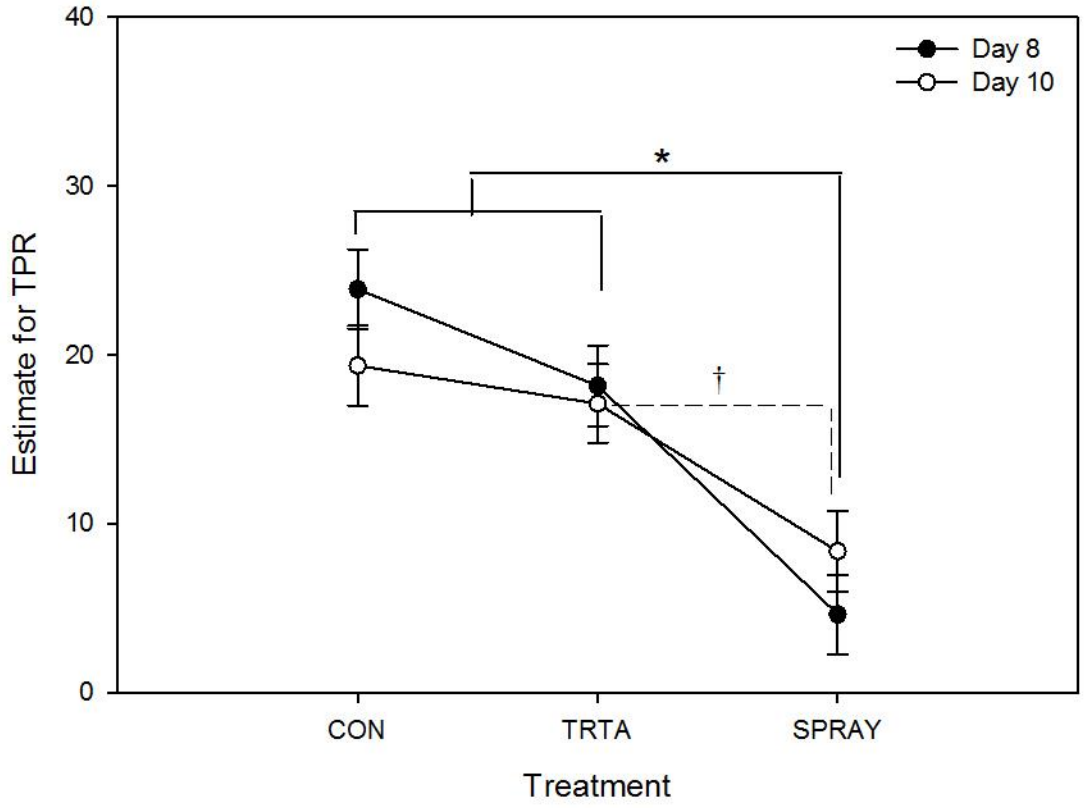


Figure 2.5. Estimate of tail paint removed (TPR) for effects of treatment, day of ultrasound, and interactions. Treatment: ($P < 0.001$). All other effects and interactions were not significant ($P < 0.20$). Estimates between days differ significantly ($P < 0.02$). †Estimates between day tend to differ ($P < 0.08$)

CHAPTER 3

EFFECTS OF RUMEN-PROTECTED METHIONINE OR CHOLINE SUPPLEMENTATION ON VAGINAL DISCHARGE AND UTERINE CYTOLOGY OF HOLSTEIN COWS

ABSTRACT

Seventy-two Holstein cows were fed the same TMR and randomly assigned to four treatments from calving to 30 DIM. Treatments were: **CON** (n = 16, fed TMR with a Lys:Met = 3.5:1), **MET** (n = 20, TMR + Smartamine M® to a Lys:Met = 2.9:1), **CHO** (n = 16, TMR + 60 g/d Reashure®), and **MIX** (n = 19; TMR Smartamine M® to a Lys:Met = 2.9:1 and 60 g/d Reashure®). Starting at d 31 cows were randomly re-assigned to two treatments: (**CON**; n = 36, TMR with a Lys:Met = 3.4:1) or (**MET**; n = 36, TMR + Smartamine M® to a Lys:Met = 2.9:1). Cows were evaluated at 4, 7, 10, 13, 15, 17, and 30 d after calving for the presence of secretion using the Metricheck® device. Contents were scored from 0 to 3 and smell was scored at 0 or 3. On 15, 30, and 72 d after calving, the uterine endometrium of all cows was sampled using a cytological brush and streaked onto slides. Each slide was counted by one person for the presence of polymorphonuclear neutrophils (**PMN**). Statistical analysis was performed using the MIXED procedure of SAS. On d 30, a treatment difference was detected using the metricheck score and smell ($P < 0.04$), with treatment MIX (0.38) having a lower score than CHO (2.11). In conclusion, supplementing cows with rumen-protected methionine may have a beneficial effect on cows' uterine health.

Key words: Methionine, choline, endometritis, metritis, PMN

INTRODUCTION

Cows usually experience negative energy balance after calving, due to low feed intake and high demands for nutrients to support milk production. The negative energy balance leads to body fat mobilization primarily and a potential excess accumulation of fat in the liver leading to fatty liver and ketosis (Komaragiri and Erdman, 1997; Drackley, 1999). Cows that develop ketosis are at risk for other metabolic disorders, have impaired reproduction, and have a high risk for developing endometritis (Reist et al., 2000; Dubuc et al., 2010). During this crucial transition period, amino acids (**AA**) are needed to export fat away from the liver in the form of very low density lipoproteins (Durand et al., 1992). An important player in the formation of very low density lipoproteins is phosphatidylcholine, which can be derived from methionine. Supplementing rumen-protected methionine has been shown to improve milk production and composition, increase DMI, reduce lipid accumulation postpartum, and promote liver function (Pisulewski et al., 1996; Ordway et al., 2009; Osorio et al., 2013). A study by Osorio et al. (2013) also reported a faster recovery rate from negative energy balance and a tendency for a lower incidence of ketosis when supplementing methionine. However, methionine is one of the most limiting AA in dairy diets (NRC, 2001) and low feed intake around the time of calving could lead to decreased synthesis of phosphatidylcholine. An alternative pathway for phosphatidylcholine is to have choline as a precursor. Choline supplementation before and after calving has been shown to reduce fatty liver and incidence of ketosis and mastitis (Lima et al., 2012).

Reducing the risk of metabolic disorders by improving liver function and increasing immune function in the transition period is key to better reproductive health. Retained placenta (**RP**), metritis, and endometritis are diseases from impaired immune function and can have

lasting negative effects on uterine health (LeBlanc, 2008). In the absence of clinical illness, metritis is defined as purulent uterine discharge within 21 d postpartum and endometritis is defined as either clinical: purulent discharge 21 d or more postpartum and mucopurulent discharge more than 26 d postpartum, or subclinical: endometrial inflammation of the uterus determined by cytology in the absence of clinical endometritis (Sheldon et al., 2006).

The majority (95%) of cows will develop metritis within the first 14 d after calving with a peak around 5 – 7 DIM, so targeting specific days within this time frame in combination with a physical exam is efficacious in diagnosing cows with metritis (Galvão, 2011). Diagnosing subclinical endometritis has been effectively done by using a cytology brush in both cows and mares and can be superior to other techniques (Kasimanickam et al., 2005; Oral et al., 2009; Defontis et al., 2011). The objective of this study was to determine the effects of feeding rumen-protected methionine and choline pre – and postpartum on reproduction of Holstein cows through the assessment of vaginal discharge and uterine cytology.

MATERIALS AND METHODS

Experimental Design and Treatments

The University of Illinois Institutional Animal Care and Use Committee (IACUC) approved all following experimental procedures. Seventy-two (n = 72) pregnant Holstein cows entering their 2nd or greater lactation were enrolled in this trial. The experimental design was a randomized complete block design. Cows were housed in tie stalls bedded with sand at the University of Illinois Dairy Cattle Research Unit (Urbana, Illinois). All cows were fed the same fresh cow diet from 0 – 30 DIM and a high cow diet from 31- 72 DIM to meet but not exceed 100% of the energy requirements as outlined by NRC 2001. At calving, cows were randomly

assigned to one of four treatments, given as a top-dress on a TMR: supplementation with rumen-protected methionine (**MET**; n = 20, received 0.08% of the DM of the diet/d as methionine, Smartamine M®, Adisseo, Alpharetta, GA, USA, to a Lys:Met = 2.9:1), rumen-protected choline (**CHO**; n = 17, received 60 g/d choline, Reassure, Balchem Corporation, New Hampton, NY), both rumen protected methionine and choline (**MIX**; n = 19, received 0.08% of the DM of the diet/d as methionine to a Lys:Met = 2.9:1 and 60 g/d choline), or no supplementation to serve as control (**CON**; n = 16, fed TMR with a Lys:Met = 3.5:1).

After calving from 30 ± 1 DIM to 72 ± 1 DIM, cows were randomly re-assigned to two new treatments: control (**CON**; n = 36, fed basal diet with a Lys:Met = 3.4:1) and methionine (**MET**; n = 36, fed basal diet plus methionine to a Lys:Met = 2.9:1). Therefore, after 30 DIM there were a total of 8 treatments: **CON-CON** (n = 6), **CON-MET** (n = 10), **MET-CON** (n = 10), **MET-MET** (n = 10), **CHO-CON** (n = 11), **CHO-MET** (n =6), **MIX-CON** (n = 9), and **MIX-MET** (n = 10). A schematic of the treatment designs for the entire study is shown in Figure 3.1.

Sample Collection

Dry matter intake was determined daily throughout the dry period and first 72 d post-calving. Body weight and body condition scores (scale of 1 = emaciated to 5 = obese; Fergunson, 1994) were obtained weekly throughout the study. BCS was assigned in quarter-unit increments by two individuals each time and the average of the score was used for that week. Health disorders included RP, displaced abomasum (**DA**), clinical ketosis, mastitis, hypocalcemia, hoof problems, and fever. Retained placenta was defined as a placenta that failed to deliver completely longer than 12 h after calving; DA was diagnosed by a veterinarian; ketosis was diagnosed by farm staff or a veterinarian by urinalysis strip (Ketostix, Bayer Corp. Diagnostics

Division, IN); mastitis was diagnosed by altered milk composition confirmed by positive microbiological culture; hypocalcemia was diagnosed by trained farm staff and veterinarians; hoof problems were defined as cows with abnormal hoof disorders that required extra hoof care such as warts, ulcers, punctures or other injury, abscesses, etc.; fever was defined as cows having a temperature of greater than 39.5°C on d 4, 7, 10, 13, 15, 17, or 30 relative to calving.

Vaginal Discharge Evaluation

Cows were evaluated at 4, 7, 10, 13, 15, 17, and 30 d after calving for the presence of vaginal secretions by inserting the a device into the vagina of the cow (Metricheck®, Simcro, New Zealand). The Metricheck® (**MC**) device consists of a 50 cm long stainless steel rod with a 4 cm rubber hemisphere tip that is used to collect vaginal contents. The MC was disinfected before each use with chlorhexidine diacetate disinfectant (Nolvasan Solution, Zoetis Animal Health, Florham Park, NJ). To minimize contamination, the tails of cows were held aside and the vulva was cleaned with Nolvasan solution and dried with paper towels. Sterile lubricant (Therio-gel, Agtech, Inc., Manhattan, KS) was applied to the convex part of the rubber tip before insertion. The MC was inserted through vulva and into the cranial portion of the vagina fornix, after which the tool was retracted at a slight upward angle to not lose any contents. The vaginal contents were examined and scored on a scale of 0 – 3: score 0 = clear or translucent mucus; score 1 = mucus containing small flecks of white or off-white pus; score 2 = discharge containing ≤ 50% white or off-white mucopurulent material; and score 3 = discharge containing ≥ 50% purulent material, usually white or yellow, but sometimes sanguineous (Sheldon et al., 2006). Immediately following externalization of the MC, the contents were also smelled and quantified (smell 0 = no odor or smell 3 = fetid odor). The MC was not used if cows had retained

placentas or other severe physical injuries to the vulva. Temperature was taken at the time of discharge evaluation.

Uterine Endometrial Cytology

Endometrial samples were collected for cytology analysis at 15, 30, and 72 d after calving using a cytology brush (Andwin Scientific, CA). The cytology brush was inserted into a sterile stainless steel rod and then placed into a stainless steel tube for passage through the cervix. The tube was placed in a sanitary plastic sleeve to prevent contamination. The vulva was washed with warm water and dried with paper towels before being sprayed and wiped with ethanol. The instrument was passed into the cervix where the plastic sleeve was punctured, and the instrument was advanced into the body of the uterus. In the uterine body, the stainless steel tube was pulled back to expose the cytology brush. Endometrial samples were collected by rotating the handle of the stylet while in contact with the uterine wall. The cytology brush was then retracted back into the stainless steel tube prior to removal from the cow. The instrument was sanitized with disinfectant or autoclaved between uses.

Slides were prepared immediately following collection by rolling the cytology brush onto clean, glass microscope slides and fixing the sample with cytofixative (Cytoprep; Fisher Scientific, Pittsburgh, PA). Slides were brought to the laboratory and stained with a Giemsa stain (Camco Quik Stain II – Self Buffered Differential Wright-Giemsa Stain, Cambridge Diagnostic Products, FL). Slides were allowed to dry for 24 h before a glass coverslip was added using a mounting medium (Permount, Fisher Scientific). Slides were scanned using whole slide imaging (NanoZoomer Digital Pathology System, Hamamatsu Photonics, Japan) at the Institute for Genomic Biology at the University of Illinois.

A minimum of 5 images were captured from 5 different representative areas on each slide (NDP.view software, Hamamatsu Photonics) using 20x magnification. A minimum of 100 total cells per slide were counted (Image J, National Institutes of Health, MD) and the percentage of epithelial cells and polymorphonuclear neutrophils (**PMN**) was determined. An example of an epithelial cell and PMN can be seen in Figure 3.2. All slides were counted by the same technician.

Statistical Analyses

All statistical analyses were performed using SAS 9.4 (SAS Institute Inc., NC). Measurements for dry matter intake and milk yield were reduced to weekly means before statistical analysis. Mixed models were created using the MIXED procedure in SAS to analyze production (BW, BCS, DMI, and milk yield), MC, and PMN variables. The model for production data contained the fixed effects of Treatment, Week, the interaction of Treatment by Week, and covariates; cow was the experimental unit and was analyzed as a random variable. Week 1 BW, BCS, and milk yield were used as covariates for BW, BCS, and milk yield and calving season was used in the model for all production variables. Covariates were not included in the model if found to be non-significant ($P > 0.05$). Week was used as a repeated measure with cow as the subject. Residual distribution was evaluated for normality and homoscedasticity. For variables with treatment differences, letters were assigned so that treatments with the same letter are not different.

The MC and PMN data were analyzed for each day sampled. This analysis used the same model as explained for the production data; however, it excluded the fixed effect of week and the interaction with treatment. The *P*-values for MC and PMN data were transformed by taking the log of the measurements for better homoscedasticity of the residuals. The *P*-values are reported

as the transformed results; however, all least squares means estimates were back-transformed for the reported results. Letters were also assigned to the MC and PMN results when applicable, so that treatments with the same letter are not different.

Logistic regression was done using the LOGISTIC procedure in SAS considering the binary response variables of metritis and for each health event and odds ratios (**OR**) were calculated. The likelihood for the response variable of metritis or endometritis was done for MC score, smell, score plus smell, and the percentage of PMN. Cows were considered to have metritis if the MC score was = 3, smell = 3, or the combination of score plus smell was ≥ 3 on d 4 – 17 and endometritis if the MC score was ≥ 2, smell = 3, or the combination of score plus smell was ≥ 2 on d 30 (Sheldon et al., 2006). Using the percentage of PMN, cows were considered to have subclinical endometritis if greater 18% on d 30 (Sheldon et al., 2006), and 5% on d 72 (Oral et al., 2009). There are no literature cutoff values reported for the percentage of PMN on d 15 so our cutoff (40%) was determined by taking the median of our data from all cows on d 15. For health data, cows were considered positive for the health event if they were diagnosed with the disorder at any time during the experiment or with a fever if they had a temperature of > 39.5ºC on d 4, 7, 10, 13, 15, 17, or 30 after calving. For all logistic analyses, the referent treatment is CON for d 0 – 30 and to CON-CON for d 31 – 72. Statistical significance was declared as *P*-value lower than 0.05, and a tendency declared as *P* value lower than 0.10.

RESULTS AND DISCUSSION

Production and Health

The ingredient composition of the diets is shown in Table 3.1a and the analyzed chemical composition of the diets is shown in Table 3.1b. Results for production variables are shown in

Table 3.2 for weeks 1 to 4 when there were four treatments, and in Table 3.3 for weeks 5 to 10 when there were eight treatments. Body weight, DMI, and milk yield were not affected by treatment ($P > 0.17$) at any time in the experiment; least squares means for BW of wk 1 to 4 = 675.8 kg (range 660.5 – 702.5 kg) and wk 5 to 10 = 640.3 kg (range 612.7 – 655.7 kg), least squares means for DMI of wk 1 to 4 = 18.2 kg (range 17.1 – 19.2 kg) and of wk 5 to 10 = 21.9 kg (range 20.4 – 23.1), and least squares means for milk yield of wk 1 to 4 = 41.1 kg/d (range 38.1 – 42.3 kg/d) and of wk 5 to 10 = 46.4 kg/d (range 44.4 – 48.3 kg/d). These results agree with a previous report by Ordway et al. (2009), which observed no difference in DMI between cows fed rumen-protected methionine (Smartamine M®) and cows not supplemented (control). On the other hand, other authors report increases (Osorio et al., 2013) and decreases (Socha et al., 2005) in DMI for cows supplemented with rumen-protected methionine. These differences are most likely from variations in Lys:Met, when supplementation started (prepartum vs. postpartum), and composition or consistency of the diet.

No treatment by week interaction was observed for DMI and milk yield ($P > 0.33$), however there was an interaction ($P = 0.05$) for BW in wk 1 to 4 (Figure 3.3) and a tendency ($P = 0.10$) in wk 5 to 10 (Figure 3.4). This interaction can most likely be explained due to changes over time and variation between cows rather than the interaction being associated with the main effect of treatment ($P > 0.45$). Body condition score was not affected by treatment ($P = 0.49$) in wk 1 to 4, but there was a difference in treatments ($P = 0.004$) for wk 5 – 10; average BCS of wk 1 to 4 = 3.41 (range 3.36 – 3.44) and of wk 5 to 10 = 3.06 (range 2.80 – 3.20). In wk 5 to 10, CON-CON had the lowest BCS of any treatment at 2.80, and CON-MET had the highest at 3.20 ($P = 0.004$). There were no differences among the other treatments.

Table 3.4 shows the incidence of the health events during the experiment for four treatments and Table 3.5 shows information for the logistic analysis and OR. Treatment MIX was the only treatment that did not have any prevalence of RP or hoof problems, but was the only treatment that had an incidence of hypocalcemia, and had the highest prevalence of fever (13.89 %). Treatment CHO had the highest prevalence of RP (4.17 %) and DA (5.56 %). Treatments CON, MET, and CHO all had the same percentage of cows with ketosis (4.17 %) and MIX had 1.39 % prevalence of ketosis. Both CON and MET also had the same prevalence of RP (2.78 %), and DA (0 %). No significant differences were found in the OR between treatments for health events. These results are in contrast with a previous report by Lima et al. (2012) that observed a significant decrease in the incidence of ketosis and mastitis compared for cows supplemented with rumen-protected choline when compared with cows not supplemented 22 d before calving to 80 d after calving.

Vaginal Discharge

The least squares means and SEM data from vaginal contents and temperature can be seen in Table 3.6. The MC score and smell were evaluated separately as well as combined into a single variable. A treatment tendency was observed for MC score on d 4 ($P = 0.08$) and difference on d 30 ($P = 0.03$). On d 4, cows that received CHO (2.75) had the lowest score and this was significantly different from treatments MET and MIX (3.00). There was no difference between treatment CON and any other treatment. However, on d 30, cows that received CHO (1.36) had the highest score and were different ($P = 0.03$) from cows that received MET (0.43) and MIX (0.11), which had the lowest scores. Again, no difference was found between CON and the other three treatments, where cows that received CON had the second highest score on d 30.

A difference was observed between treatments for smell on d 7 ($P = 0.005$), d 10 ($P = 0.003$), d 17 ($P < 0.0001$) and d 30 ($P = 0.04$). On d 7 and d 10, no difference was observed between cows that received MET and CHO, and these groups had a higher smell score when compared with CON and MIX. However, on d 17 and d 30, cows that received MET were no longer different from those that received CON and MIX, with all three groups having a smell score of 0 and the CHO group much higher with a score of 0.67 (Table 3.6). When the MC score and smell was combined into a single score, the results were similar to just score alone, with differences detected on d 4 and d 30 ($P < 0.07$); however, on d 30 only a difference between treatments CHO and MIX was detected (Table 3.6).

Tables 3.7a to 3.7c show logistic analysis for the likelihood of cows having metritis/endometritis based on MC score, smell, and score + smell. No differences were found in the OR for score or score + smell. Smell alone, a difference ($P = 0.08$) was observed in the OR for d 7 where MET was more likely to have a metritis when compared to CON (OR = 7.64). Although a treatment difference was observed between scores and smell, no differences were found between treatments for temperature on any day ($P > 0.13$). This supports the hypothesis that a fever does not always accompany fetid and/or purulent discharge and diagnosis of metritis should consist of factors in addition to temperature (Benzaquen et al., 2007).

These differences between d 4 and d 30 for score and score + smell may be somewhat explained with the incidence of health events. On d 4 treatment CHO had the lowest score and score + smell, which might be in part because cows that received CHO had a higher incidence of RP. It has been reported that if RP occurs, the membrane is retained on average for 7 d (Eiler, 1997). Metricheck was not performed if cows still had retained membranes so most likely there were less scores reported for cows in the CHO treatment on d 4, which may account for the

difference when compared with other treatments. Likewise, treatment MIX had the lowest score and score + smell by d 30 and did not have any incidence of RP, and treatments CON and MET had similar RP incidence and similar scores and score + smell. The higher occurrence of RP in treatment CHO may also account for the change from the lowest score on d 4 to the highest by d 30 and the higher smell score on d 17 and 30, since RP is a risk factor for metritis and endometritis. Cows that received MET had significantly higher smell than CON or MIX and even numerically higher than CHO on d 7 and 10, but by d 17 MET was the same as CON and MIX, with a smell of 0. Fetid odor has been associated with a greater load of bacteria such as *A. pyogenes*, which has been associated with purulent discharge, subsequent endometritis, and impaired reproductive performance (LeBlanc, 2008; Sheldon et al., 2006). Treatment MET was able to "resolve" the smell by d 30 but CHO was not. Therefore, supplementing methionine early postpartum may be beneficial to cows challenged with bacterial uterine infections.

Uterine Cytology

Data for the percentage of PMN are shown in Table 3.8. Examples of images from cows with percentage of PMN close to these cutoff values can be seen in Figure 3.3. For all three days, no significant difference between treatments was detected. On d 15 and d 30, treatment MIX numerically had a much higher percentage of PMN when compared to the other three treatments and was significantly more likely (OR = 5.60; $P < 0.05$) to have endometritis on d 30 when compared with CON (Table 3.9). Additionally, the function of PMN is associated with the risk of RP, metritis, and endometritis (Hammon et al., 2006; Kimura et al., 2006). In the present study, we did not observe cows with RP in the treatment MIX, significantly higher MC score at d 4 but lower MC scores at d 30, and numerically higher PMN early on but lower PMN on d 72. It may be possible that a higher percentage of PMN early postpartum may be indicative of a greater

immune response. Supplementation with MIX may enhance the immune response. For all treatments, with the exception of MIX-CON and MIX-MET, the treatments that received MET after 30 d had numerically lower percentage of PMN by d 72 (i.e. CON-MET was lower than CON-CON). Continuing to supplement with methionine after d 30 may be beneficial to long-term uterine health.

IMPLICATIONS

Supplementation with methionine in the postpartum period has varying effects on production data in literature and no differences were observed for production data in the present study. Significant treatment differences were observed in MC score, smell, and a combined score plus smell. Cows that received MIX had no incidence of RP, had significantly lower MC scores on d 30 when compared with CHO, and had numerically lower percentage of PMN on d 72 when compared with all other treatments. Rumen-protected methionine may be beneficial to uterine immune response and long-term reproductive health. This improvement may be even greater when methionine is used in combination with choline supplementation.

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TABLES AND FIGURES

Table 3.1a. Ingredient composition of diets fed to cows throughout the experiment on a % DM basis

	0-30 DIM	31-72 DIM
Alfalfa silage	5.07	6.12
Alfalfa hay	2.98	6.94
Corn silage	33.41	35.09
Wheat straw	2.98	-
Cottonseed	3.58	3.26
Wet brewers grains	9.09	8.16
Ground shelled corn	23.87	25.09
Soy hulls	4.18	4.74
Soybean meal, 48% CP	2.39	2.45
Expeller Soybean meal[2]	5.97	1.22
ProVAAl® AAdvantage[3]	1.50	1.43
Urea	0.18	0.33
Rumen-inert fat[1]	1.02	1.43
Limestone	1.31	1.14
Salt	0.30	0.30
Dicalcium phosphate	0.30	0.30
Magnesium oxide	0.12	0.12
Sodium bicarbonate	0.79	0.78
Potassium carbonate	0.30	0.30
Calcium sulfate	0.12	0.12
Mineral vitamin mix	0.18	0.53
Biotin	0.35	-

[1]Energy Booster 100 (Milk Specialties Global, Eden Prairie, MN)

[2]SoyPLUS (West Central Cooperative, Ralston IA)

[3]Perdue AgSolutions LLC, Binghamton, NY

Table 3.1b. Mean chemical composition and SD of diet fed throughout the experiment on a DM basis

Component	0-30 DIM	31-72 DIM
CP, %	16.8 ± 0.77	16.18 ± 0.51
ADF, %	22.0 ± 2.1	22.96 ± 1.62
NDF, %	34.6 ± 2.9	34.01 ± 2.62
Lignin, %	3.5 ± 0.45	3.85 ± 0.78
NFC, %	33.9 ± 2.9	35.53 ± 2.45
Starch, %	22.2 ± 0.1	24.78 ± 2.28
Crude fat, %	5.46 ± 0.24	5.86 ± 0.33
Ash, %	9.2 ± 1.4	8.39 ± 0.61
TDN[1], %[2]	71.2 ± 2.0	72.15 ± 1.46
NE_l, Mcal/kg[2]	0.76 ± 0.02	0.78 ± 0.01
Calcium, %	1.57 ± 0.57	1.26 ± 0.21
Phosphorus, %	0.38 ± 0.03	0.36 ± 0.04
Magnesium, %	0.28 ± 0.03	0.27 ± 0.04
Potasium, %	1.21 ± 0.15	1.28 ± 0.19
Sodium, %	0.34 ± 0.04	0.39 ± 0.06
Iron, PPM[3]	636.1 ± 220.7	494.15 ± 80.81
Zinc, PPM	92.4 ± 9.56	87.77 ± 17.55
Copper, PPM	19 ± 3.3	17.46 ± 2.76
Manganese, PPM	101.1 ± 26.0	91.23 ± 12.79
Molybdenum, PPM	0.88 ± 0.13	0.82 ± 0.16
Sulfur, %	0.24 ± 0.03	0.23 ± 0.03

[1]TDN: Total digestible nutrients
[2]Calculated according to NRC, 2001.
[3]PPM: Part per million

Table 3.2. Least squares means and SEM for production data for weeks 1-4 using 4 treatments (TRT)

Variable	Treatment[1]				SEM	*P*-Value[2]		
	CON	MET	CHO	MIX		TRT	wk	TRT*wk
Body weight, kg[3]	660.48	702.51	674.28	666.07	8.11	0.45	<0.0001	0.05
Body condition score[4]	3.42	3.36	3.44	3.42	0.04	0.49	<0.0001	0.91
Dry matter intake, kg	17.95	18.64	17.09	19.22	0.84	0.36[3]	<0.0001	0.33
Milk yield, kg	41.59	42.27	38.05	42.34	1.47	0.17[3]	<0.0001	0.92
Fat, %	3.11[a]	3.28[b]	3.19[ab]	3.31[b]	0.05	0.02	<0.0001	0.63
Fat, kg	1.26[ab]	1.38[a]	1.20[b]	1.38[a]	0.05	0.03[3]	0.008	0.62
Protein, %	3.40	3.60	3.50	3.53	0.10	0.61[3]	<0.0001	0.48
Protein, kg	1.38	1.52	1.37	1.47	0.05	0.19[3]	<0.0001	0.95
Lactose, %	4.81[a]	4.69[b]	4.66[b]	4.73[ab]	0.04	0.06	<0.0001	0.17
Lactose, kg	2.01	1.99	1.80	2.01	0.08	0.21[3]	<0.0001	0.92
Non-fat solids, %	5.72	5.61	5.58	5.66	0.04	0.10	<0.0001	0.19
Total solids, %	12.16	12.66	12.50	12.55	0.15	0.17[3]	<0.0001	0.64
Milk urea nitrogen, mg/dL	13.09	12.73	13.24	12.87	0.39	0.82[3]	0.34	0.54
Somatic cell count	97.97	87.68	101.34	171.07	29.00	0.20	0.004	0.31

[1]Treatments: CON: control, n=16; MET: methionine, n=20; CHO: choline, n=17; MIX: methionine and choline, n=19
[2]Calving season was used as a covariate (P <0.05)
[3]Body weight for week 1 was used as a covariate (P <0.002)
[4]Body condition score for week 1 was used as a covariate (P <0.0001)
[a-b]Means within a row with different superscripts differ significantly

Table 3.3. Least squares means and standard error of the mean (SEM) for production data for weeks 5-10 using 8 treatments (TRT)

Variable	Treatment[1]								SEM	*P*-Value[2]		
	CON-CON	CON-MET	MET-CON	MET-MET	CHO-CON	CHO-MET	MIX-CON	MIX-MET		TRT	wk	TRT*wk
Body weight, kg	612.77	644.98	655.74	648.39	628.37	635.58	651.38	645.64	9.79	0.89	0.59	0.10
Body condition score	2.80[a]	3.20[b]	3.09[bc]	3.01[c]	3.07[bc]	3.12[bc]	3.03[c]	3.16[bc]	0.06	0.004	0.03	0.30
Dry matter intake, kg	22.98	22.04	21.05	23.16	21.49	20.39	20.85	23.17	0.89	0.30[3]	<0.0001	0.69
Milk yield, kg	47.52	44.47	47.51	47.14	46.22	44.40	45.74	48.29	2.07	0.89[3]	<0.0001	0.74
Fat, %	2.49	2.54	2.65	2.67	2.90	2.60	2.70	2.73	0.10	0.33	<0.0001	0.05
Fat, kg	1.24	1.19	1.27	1.29	1.35	1.18	1.35	1.33	0.05	0.38	<0.0001	0.002
Protein, %	2.60	2.74	2.68	2.78	2.68	2.64	2.66	2.80	0.05	0.22	0.0007	0.004
Protein, kg	1.27	1.27	1.30	1.34	1.26	1.21	1.32	1.36	0.05	0.63	0.02	0.06
Lactose, %	4.82	4.86	4.81	4.77	4.92	4.74	4.76	4.83	0.04	0.12[3]	0.01	0.60
Lactose, kg	2.32	2.27	2.33	2.30	2.35	2.19	2.40	2.36	0.09	0.97	<0.0001	0.09
Non-fat solids, %	5.69	5.73	5.71	5.67	5.80	5.64	5.67	5.72	0.04	0.35	0.009	0.64
Total solids, %	10.57	10.97	11.05	11.10	11.23	10.60	11.04	11.16	0.16	0.22[3]	<0.0001	0.11
Milk urea nitrogen, mg/dL	11.57	11.93	12.21	12.81	13.27	12.72	13.33	13.27	0.54	0.36[3]	0.34	0.28
Somatic cell count	20.19	98.02	161.14	141.69	41.18	73.79	207.05	288.16	78.38	0.45[3]	0.93	0.28

[1]Treatments: CON-CON: control then control, n=6; CON-MET: control then methionine, n=10; MET-CON: methionine then control, n=10; MET-MET: methionine then methionine, n=10; CHO-CON: choline then control, n= 11; CHO-MET: choline then methionine, n=6; MIX-CON: methionine and choline then control, n=9; MIX-MET: methionine and choline then methionine, n=10

[2]Calving season was used as a covariate when significant (P <0.05)

[a-c]Means within a row with different superscripts differ significantly

Table 3.4. Health events for cows throughout trial

Variable	Treatment[1] % (n/n)				Total % (n/n)
	CON	MET	CHO	MIX	
Retained placenta	2.78 (2/72)	2.78 (2/72)	4.17 (3/72)	0 (0/0)	9.72 (7/72)
Displaced abomasum	0 (0/0)	0 (0/0)	5.56 (4/72)	1.39 (1/72)	6.94 (5/72)
Ketosis	4.17 (3/72)	4.17 (3/72)	4.17 (3/72)	1.39 (1/72)	13.89 (10/72)
Hypocalcemia (milk fever)	0 (0/0)	0 (0/0)	0 (0/0)	1.39 (1/72)	1.39 (1/72)
Mastitis	0 (0/0)	2.78 (2/72)	1.39 (1/72)	0 (0/0)	4.17 (3/72)
Hoof problems	1.39 (1/72)	4.17 (3/72)	1.39 (1/72)	0 (0/0)	6.94 (5/72)
Fever[2]	6.94 (5/72)	11.11 (8/72)	9.72 (7/72)	13.89 (10/72)	41.67 (30/72)

[1]Treatments: CON: control, n=16; MET: methionine, n=20; CHO: choline, n=17; MIX: methionine and choline, n=19
[2]Temperature of 39.5 C or greater on d 4, 7, 10, 13, 15, 17, or 30

Table 3.5. Logistic analysis for likelihood of cows developing health disorders in each treatment

Event	n	Level[1]	Coefficient	SEM	Odds ratio	95% CI	*P*-Value
Retained placenta	72	MET	–0.25	1.06	0.78	–2.33 – 1.83	0.81
		CHO	0.41	0.99	1.50	–1.53 – 2.34	0.68
		MIX	–11.45	185.60	<0.01	–375.22 – 352.32	0.95
Displaced abomasum	72	MET	–9.82	192.96	1.00	–261.96 – 242.32	1.00
		CHO	11.53	143.82	>999	–270.36 – 293.42	0.94
		MIX	9.82	143.83	>999	–272.08 – 291.72	0.95
Ketosis	72	MET	–0.27	0.90	0.77	–2.02 – 1.49	0.76
		CHO	–0.07	0.90	0.93	–1.84 – 1.70	0.93
		MIX	–1.42	1.21	0.24	–3.80 – 0.95	0.24
Hypocalcemia	72	MET	–0.45	224.78	0.64	–441.00 – 440.11	1.00
		CHO	–0.45	235.55	0.64	–462.11 – 461.21	1.00
		MIX	9.90	149.79	>999	–281.68 – 303.49	0.95
Mastitis	72	MET	11.01	184.06	>999	–349.74 – 371.75	0.95
		CHO	10.43	184.06	>999	–350.31 – 371.17	0.95
		MIX	<0.001	249.81	1.00	–489.61 – 489.61	1.00
Hoof problems	72	MET	0.97	1.21	2.65	–1.39 – 3.34	0.42
		CHO	–0.06	1.46	0.94	–2.92 – 2.80	0.96
		MIX	–11.00	217.60	<0.01	–437.50 – 415.49	0.96
Fever[2]	441	MET	–0.03	0.46	0.97	–0.93 – 0.87	0.94
		CHO	–0.15	0.50	0.86	–1.12 – 0.83	0.77
		MIX	0.22	0.44	1.24	–0.64 – 1.07	0.62

[1]Referent is CON. Treatments: CON: control, n=16; MET: methionine, n=20; CHO: choline, n=17; MIX: methionine and choline, n=19

[2]Cutoff was a temperature of 39.5 C or greater on d 4, 7, 10, 13, 15, 17, or 30

Table 3.6. Treatment least squares means and SEM of each day for metricheck traits

			Treatment[1]					
Variable	Day	n	CON	MET	CHO	MIX	SEM	*P*-Value[2,3]
Score	4	48	2.92^{ab}	3.00^{a}	2.75^{b}	3.00^{a}	0.06	0.08
	7	67	2.30	2.21	2.53	2.04	0.23	0.42[3]
	10	60	2.93	2.88	3.00	2.81	0.07	0.19
	13	64	2.51	2.49	2.26	2.30	0.24	0.51[3]
	15	69	2.50	2.53	2.27	2.21	0.23	0.17
	17	70	2.06	2.37	2.38	1.74	0.23	0.29
	30	47	0.65^{ab}	0.43^{a}	1.36^{b}	0.11^{a}	0.29	0.03[3]
Smell	4	56	0	0.75	0.75	0	0.24	0.14
	7	62	0^{a}	1.38^{b}	0.94^{b}	0.47^{a}	0.27	0.005
	10	64	0^{a}	1.17^{b}	0.94^{b}	0^{a}	0.25	0.003
	13	64	0.60	1.06	1.00	1.06	0.35	0.77
	15	69	0.75	0.95	1.40	0.47	0.31	0.26
	17	65	0^{a}	0^{a}	0.75^{b}	0^{a}	0.16	<0.0001
	30	45	0^{a}	0^{a}	0.67^{b}	0^{a}	0.16	0.04
Temperature	4	58	38.50	38.53	38.34	38.51	0.09	0.52
	7	66	38.59	38.62	38.59	38.59	0.10	0.97
	10	67	38.75	38.59	38.60	38.60	0.09	0.63[3]
	13	63	38.34	38.35	38.67	38.57	0.11	0.13
	15	68	38.35	38.41	38.40	38.54	0.10	0.63[3]
	17	65	38.46	38.45	38.61	38.49	0.08	0.50
	30	46	38.39	38.23	38.29	38.63	0.14	0.25
Score + smell	4	55	2.57^{ab}	3.50^{a}	1.67^{b}	3.25^{a}	0.32	0.07
	7	67	2.53	3.41	3.50	2.58	0.43	0.29
	10	69	3.13	3.94	3.50	2.79	0.40	0.30
	13	64	3.13	3.65	3.33	3.35	0.48	0.89
	15	69	3.25	3.47	3.67	2.68	0.46	0.67
	17	70	2.06	2.84	3.13	2.05	0.37	0.15
	30	47	1.15^{ab}	1.08^{ab}	2.11^{a}	0.38^{b}	0.40	0.04

[1]Treatments: CON: control, n=16; MET: methionine, n=20; CHO: choline, n=17; MIX: methionine and choline, n=19

[2]*P*-Values have been log transformed

[3]Calving season was used as a covariate when significant ($P < 0.05$)

[a-b]Means within a row with different superscripts differ significantly

Table 3.7a. Logistic analysis for likelihood of cows having metritis based on a metricheck score of 3 on d 4 - 17, or endometritis based on a score ≥ 2 on d 30

Day	n	Level[1]	Coefficient	SEM	Odds ratio	95% CI	*P*-Value
4	58	MET	0.65	1.00	1.91	–1.31 – 2.60	0.52
		CHO	–1.30	0.87	0.27	–3.01 – 0.41	0.14
		MIX	0.65	1.00	1.91	–1.31 – 2.60	0.52
7	67	MET	–0.09	0.75	0.92	–1.55 – 1.38	0.91
		CHO	0.10	0.77	1.10	–1.41 – 1.60	0.90
		MIX	–0.15	0.73	0.86	–1.58 – 1.27	0.83
10	69	MET	0.14	0.90	1.15	–1.62 – 1.91	0.87
		CHO	<0.0001	0.91	1.00	–1.78 – 1.78	1.00
		MIX	–0.69	0.81	0.50	–2.28 – 0.89	0.39
13	64	MET	0.15	0.91	1.17	–1.62 – 1.93	0.86
		CHO	–0.69	0.85	0.50	–2.35 – 0.97	0.41
		MIX	–1.03	0.81	0.36	–2.62 – 0.56	0.20
15	69	MET	–0.33	0.76	0.72	–1.81 – 1.16	0.67
		CHO	–0.41	0.80	0.67	–1.96 – 1.15	0.61
		MIX	–0.99	0.74	0.37	–2.44 – 0.45	0.18
17	70	MET	–0.15	0.68	0.86	–1.48 – 1.19	0.83
		CHO	0.54	0.74	1.71	–0.91 – 1.98	0.47
		MIX	–1.02	0.71	0.36	–2.41 – 0.36	0.15
30	47	MET	–0.41	1.02	0.67	–2.40 – 1.59	0.69
		CHO	0.51	0.97	1.67	–1.38 – 2.40	0.60
		MIX	–1.28	1.23	0.28	–3.70 – 1.13	0.30

[1]Referent is CON. Treatments: CON: control, n=16; MET: methionine, n=20; CHO: choline, n=17; MIX: methionine and choline, n=19

Table 3.7b. Logistic analysis for likelihood of cows having metritis on d 4 - 17 or endometritis on d 30, based on a smell score of 3

Day	n	Level[1]	Coefficient	SEM	Odds ratio	95% CI	*P*-Value
4	58	MET	11.87	174.70	>999	–330.54 – 354.27	0.95
		CHO	11.87	174.70	>999	–330.54 – 354.27	0.95
		MIX	11.02	174.70	>999	–331.39 – 353.43	0.95
7	67	MET	2.03	1.15	7.64	–0.23 – 4.29	0.08
		CHO	1.85	1.17	6.36	–0.74 – 2.51	0.11
		MIX	0.97	1.21	2.63	–1.41 – 3.34	0.43
10	69	MET	1.01	0.80	2.76	–0.56 – 2.59	0.21
		CHO	0.68	0.84	1.97	–0.96 – 2.32	0.42
		MIX	–0.67	0.98	0.51	–2.60 – 1.26	0.49
13	64	MET	0.78	0.82	2.18	–0.83 – 2.39	0.34
		CHO	0.69	0.85	2.00	–0.97 – 2.35	0.41
		MIX	0.78	0.82	2.18	–0.83 – 2.39	0.34
15	69	MET	0.33	0.76	1.39	–1.16 – 1.81	0.67
		CHO	0.97	0.78	2.63	–0.55 – 2.48	0.21
		MIX	–0.58	0.85	0.56	–2.25 – 1.10	0.50
17	70	MET	11.46	177.80	>999	–337.01 – 359.93	0.95
		CHO	12.04	177.80	>999	–336.44 – 360.51	0.95
		MIX	10.99	177.80	>999	–337.48 – 359.47	0.95
30	47	MET	0.09	1.47	1.09	–2.80 – 2.98	0.95
		CHO	1.23	1.31	3.43	–1.34 – 3.81	0.35
		MIX	–11.03	238.85	<0.001	–479.17 – 457.11	0.96

[1]Referent is CON. Treatments: CON: control, n=16; MET: methionine, n=20; CHO: choline, n=17; MIX: methionine and choline, n=19

Table 3.7c. Logistic analysis for likelihood of cows having metritis based on the combination of a metricheck score plus smell ≥ 3 on d 4 – 17 or endometritis based on score plus smell ≥ 2 on d 30

Day	n	Level[1]	Coefficient	SEM	Odds ratio	95% CI	*P*-Value
4	58	MET	0.65	1.00	1.91	–1.31 – 2.60	0.52
		CHO	–1.30	0.87	0.27	–3.01 – 0.41	0.14
		MIX	0.65	1.00	1.91	–1.31 – 2.60	0.52
7	67	MET	0.18	0.76	1.20	–1.31 – 1.68	0.81
		CHO	0.10	0.77	1.10	–1.41 – 1.60	0.90
		MIX	–0.15	0.73	0.86	–1.58 – 1.27	0.83
10	69	MET	0.14	0.90	1.15	–1.62 – 1.91	0.87
		CHO	<0.0001	0.91	1.00	–1.78 – 1.78	1.00
		MIX	–0.69	0.81	0.50	–2.28 – 0.89	0.39
13	64	MET	0.63	0.99	1.88	–1.32 – 2.57	0.53
		CHO	–0.69	0.85	0.50	–2.35 – 0.97	0.41
		MIX	–0.78	0.82	0.46	–2.39 – 0.83	0.34
15	69	MET	–0.33	0.76	0.72	–1.81 – 1.16	0.67
		CHO	–0.41	0.80	0.67	–1.96 – 1.15	0.61
		MIX	–0.99	0.74	0.37	–2.44 – 0.45	0.18
17	70	MET	–0.15	0.68	0.86	–1.48 – 1.19	0.83
		CHO	0.54	0.74	1.71	–0.91 – 1.98	0.47
		MIX	–1.02	0.71	0.36	–2.41 – 0.36	0.15
30	47	MET	0.11	0.94	1.11	–1.73 – 1.94	0.91
		CHO	0.98	0.94	2.67	–0.86 – 2.82	0.30
		MIX	–1.28	1.23	0.28	–3.69 – 1.13	0.30

[1]Referent is CON. Treatments: CON: control, n=16; MET: methionine, n=20; CHO: choline, n=17; MIX: methionine and choline, n=19

Table 3.8. Treatment least squares means and SEM for percentage of polymorphonuclear neutrophils (PMN) in uterine endometrium cytology samples

		Treatment[1]									
Day	n	CON		MET		CHO		MIX		SEM	*P*-value[2,3]
15	62	41.96		37.77		45.76		48.01		7.18	0.97
30	70	10.15		10.30		12.61		18.31		3.93	0.22[3]
		CON-CON	CON-MET	MET-CON	MET-MET	CHO-CON	CHO-MET	MIX-CON	MIX-MET		
72	69	15.76	9.58	16.30	5.86	6.37	4.06	2.50	2.77	4.74	0.46[3]

[1]Treatments: CON: control, n=16; MET: methionine, n=20; CHO: choline, n=17; MIX: methionine and choline, n=19; CON-CON: control then control, n=6; CON-MET: control then methionine, n=10; MET-CON: methionine then control, n=10; MET-MET: methionine then methionine, n=10; CHO-CON: choline then control, n= 11; CHO-MET: choline then methionine, n=6; MIX-CON: methionine and choline then control, n=9; MIX-MET: methionine and choline then methionine, n=10

[2]*P*-values have been log transformed.

[3]Calving season was used as a covariate when significant ($P < 0.03$)

Table 3.9. Logistic analysis for likelihood of cows having subclinical endometritis based on the percentage of polymorphonuclear neutrophils (PMN) in uterine endometrium cytology samples on d 15, 30, and 72[1]

Day	n	Level[2]	Coefficient	SEM	Odds ratio	95% CI	*P*-value
15	62	MET	–0.59	0.71	0.56	–1.97 – 0.80	0.41
		CHO	0.15	0.75	1.17	–1.31 – 1.62	0.84
		MIX	<0.0001	0.73	1.00	–1.43 – 1.43	1.00
30	70	MET	0.62	0.94	1.87	–1.22 – 2.47	0.51
		CHO	0.41	0.99	1.50	–1.53 – 2.34	0.68
		MIX	1.72	0.89	5.60	–0.03 – 3.47	0.05
72	69	CON-MET	1.39	1.28	4.00	–1.13 – 3.90	0.28
		MET-CON	0.92	1.30	2.50	–1.64 – 3.47	0.48
		MET-MET	0.22	1.35	1.25	–2.42 – 2.87	0.87
		CHO-CON	1.05	1.26	2.86	–1.42 – 3.52	0.41
		CHO-MET	0.92	1.40	2.50	–1.82 – 3.65	0.51
		MIX-CON	–0.34	1.53	0.71	–3.34 – 2.66	0.83
		MIX-MET	–0.59	1.52	0.56	–3.57 – 2.39	0.70

[1]Cutoff values were 40% on d 15, 18% on d 30 and 5% on d 72

[2]Referent is CON. Treatments: CON: control, n=16; MET: methionine, n=20; CHO: choline, n=17; MIX: methionine and choline, n=19; CON-CON: control then control, n=6; CON-MET: control then methionine, n=10; MET-CON: methionine then control, n=10; MET-MET: methionine then methionine, n=10; CHO-CON: choline then control, n= 11; CHO-MET: choline then methionine, n=6; MIX-CON: methionine and choline then control, n=9; MIX-MET: methionine and choline then methionine, n=10

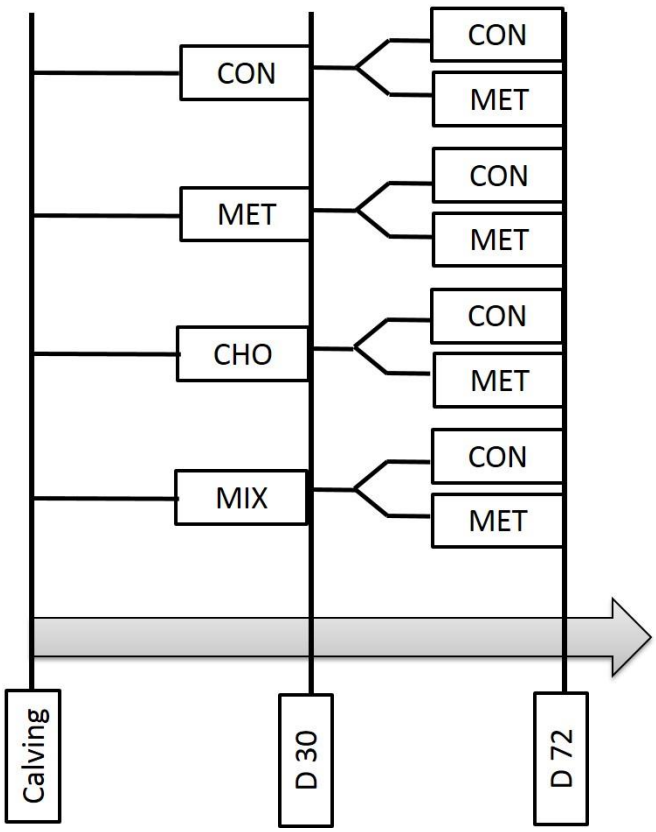


Figure 3.1. Schematic of treatment design in chronologic order through the experiment

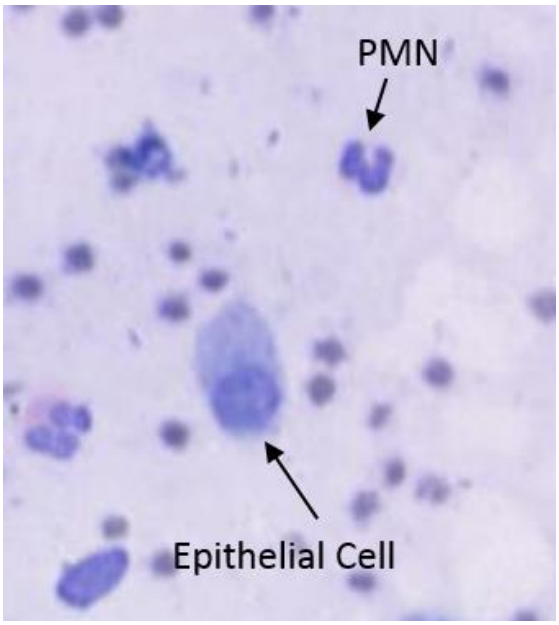


Figure 3.2. Epithelial cell compared to a PMN

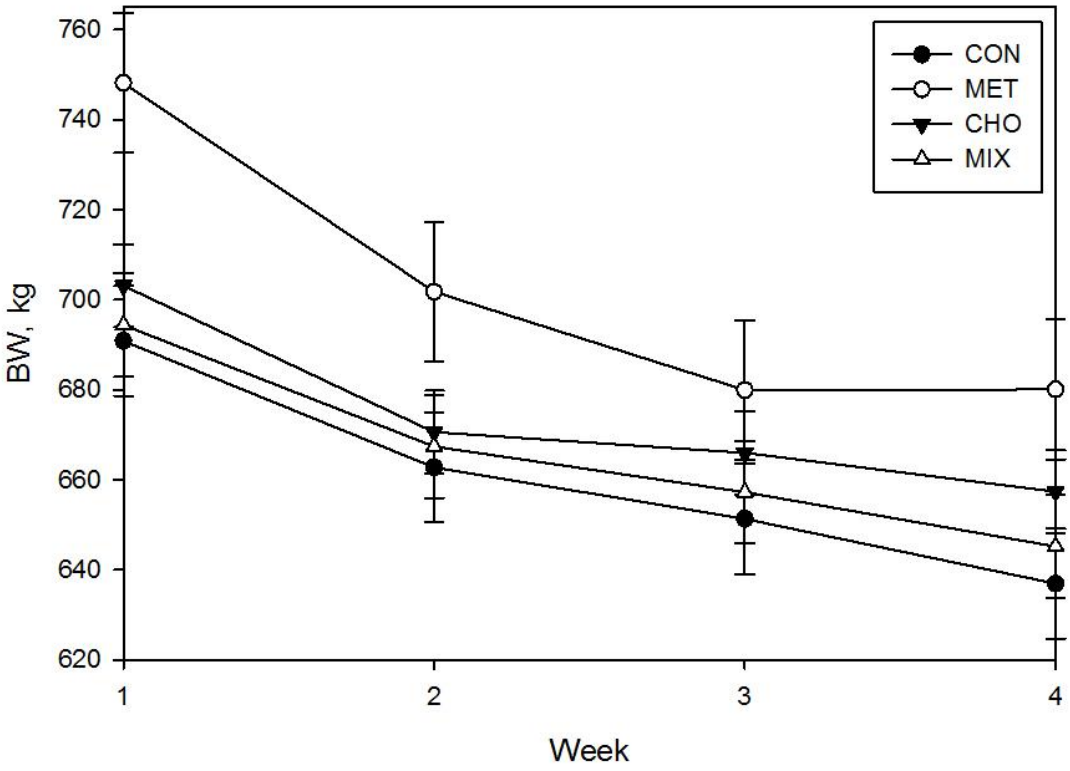


Figure 3.3. Least squares means and SEM for body weight during weeks 1-4. Treatments: CON: control, n=16; MET: methionine, n=20; CHO: choline, n=17; MIX: methionine and choline, n=19. Interaction of treatment and week *P*=0.05.

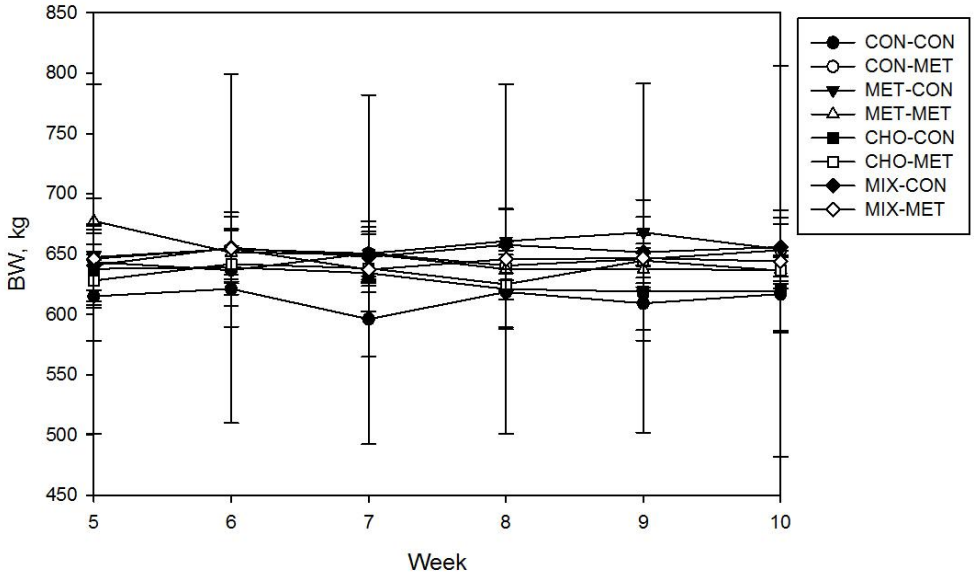
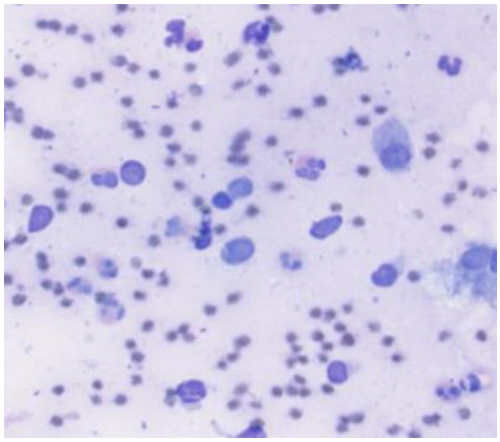
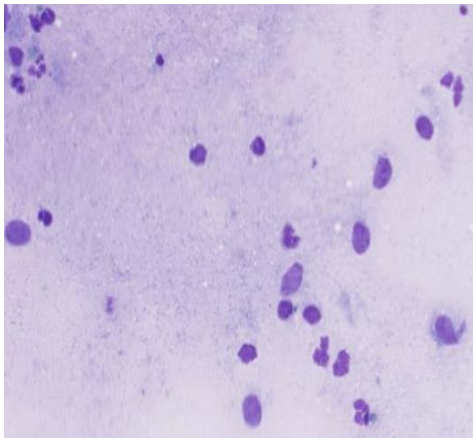


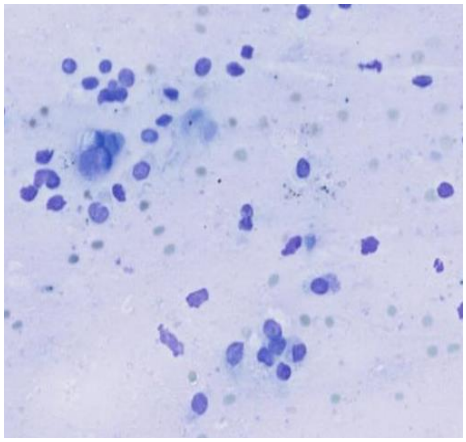
Figure 3.4. Least squares means and SEM for body weight (BW) during weeks 5-10. Treatments: CON-CON: control then control, n=6; CON-MET: control then methionine, n=10; MET-CON: methionine then control, n=10; MET-MET: methionine then methionine, n=10; CHO-CON: choline then control, n= 11; CHO-MET: choline then methionine, n=6; MIX-CON: methionine and choline then control, n=9; MIX-MET: methionine and choline then methionine, n=10. Interaction of treatment and week *P*=0.10.



D 15, cow 8855, treatment: CON
47.4% PMN
D 15 cutoff: 40%



D 30, cow 8821, treatment: CON
20.7% PMN
D 30 cutoff: 18%



D 72, cow 8770, treatment: MET-MET
5.6% PMN
D 72 cutoff' 5%

Figure 3.5. Examples of the percentage of polymorphonuclear neutrophils (PMN) found from d 15, 30, and 72

CHAPTER 4

OVERALL SUMMARY AND CONCLUSIONS

The overall objective of this thesis was to evaluate different techniques to improve reproduction on dairy farms. Specifically, we wanted to do this by comparing behaviors associated with 3 different types of tail paint formulations in Holstein heifers and determining the effects of feeding rumen-protected methionine and/or rumen-protected choline on the reproduction of Holstein cows.

In Chapter 2 we learned more about heat detection in heifers and how different tail paints were affected by behaviors, such as licking. We concluded that dairy operations that have problems with tail paint removal and false-positives may benefit from changing to a tail paint product with a different consistency, such as a spray formulation. We also observed many behaviors around estrus and periods of high and low interaction. We concluded that producers observing behaviors for heat detection can focus on heifers receiving rump lick, chin resting, anogenital sniff, mount, and attempt to mount. However, caution must be used when observing licking, chin resting and anogenital sniff since they can also be performed in non-estrus stages. Likewise, heifers that initiate mounts, attempt to mount, or push/nudge other heifers should also be considered for breeding and may be estrual or pre-estrual. Lastly, we looked at another estrus detection aid in the form of accelerometers and concluded that producers can make use of activity monitors and should focus breeding efforts on heifers that have increased standing times.

In chapter 3 we fed rumen-protected methionine and choline to postpartum Holstein cows and evaluated their reproductive health by assessing vaginal discharge through metricheck score and smell, and uterine cytology samples through the percentage of PMN. We observed

significant differences in metricheck score, smell, and a combined score + smell. We also observed cows that received both methionine and choline had no incidence of RP, significantly lower metricheck scores on d 30 when compared to choline alone, and numerically lower percentage of PMN on d 72 when compared to all other treatments. Finally, we observed cows that received methionine after 30 DIM had numerically lower percentage of PMN than cows that received the control. We concluded that rumen-protected methionine in the pre- and postpartum period may be beneficial to uterine immune response and long-term reproductive health and that this improvement may be even greater when methionine is used in combination with choline supplementation.

Overall we were able to find techniques that may be beneficial to the modern dairy industry. Better estrus detection in heifers can greatly improve reproductive efficiency and save costs from feed and missed milk yield. Using rumen-protected methionine and choline in the transition period can improve the uterine health, decrease the incidence of reproductive diseases like retained placenta and endometritis, and improve fertility. The use and combination of techniques in this thesis may improve reproductive performance across dairy farms and have a huge impact in profitability.

APPENDIX A

THE USE OF IMMUNOASSAYS FOR DAIRY COWS

In the past, radioimmunoassays (**RIA**) have been the preferred method for quantifying steroid hormones in bovine serum. These have been validated and are considered the "gold-standard" for hormone analysis. However, concerns about the safety of working with radioactive materials and the inconvenience of up keeping licenses and compliance have started to push people away from using these assays. In the dairy industry, labs that have stopped using RIA have begun sending samples out for analysis and have faced new challenges. These challenges include shipping costs and preparation and lag time while waiting on other labs to get samples done quickly and accurately. More recently, enzyme-linked immunoassays (**EIA**) have gained popularity due their ease of use and safety. These do not require the use of radioactive materials and can be done in house.

To date, comparisons have not been conducted between RIA and EIA for progesterone (**P4**) and estradiol (**E2**) levels in bovine serum. Furthermore, there are also limited numbers of EIA kits on the market that are specifically designed for the assessment of P4 and E2 in bovine serum. The goal of this study was to validate EIA kits to be used in the quantification of P4 and E2 in bovine serum and to compare the performance of several EIA kits to the historical gold standard technique, RIA. Validation of commercially available EIA kits for assessment of bovine P4 and E2 is necessary so that the industry has a standardized approach to the analysis of these steroid hormones. A standardized analysis method will yield accurate results that can be compared across various studies. In this study, for each hormone a single antibody (**SAb**) and double antibody (**DAb**) kit were compared with an RIA that used I-125 labeled hormone (n = 3 kits per hormone). All kits required modifications to the kit protocols to achieve validation.

Modifications included: serum sample extractions, making a new standard curve, increase in sample volumes, and differing incubation times. Additionally, there is a difference in ease of use between SAb and DAb kits. As the antibody-antigen binding begins as soon as the primary antibody is added, timing was critical with the SAb kit and long lag times when setting up a SAb plate had a large effect on the coefficient of variation (**CV**).

For each EIA, a custom curve was made to fit the bovine hormone profile. All samples must be in the same media, so since we were working with serum samples, our standards and buffers were made with charcoal stripped serum (serum that is stripped of all hormones by using charcoal). After checking the curve for each of the kits, we ran parallelism. Parallelism is done by taking 3 to 4 samples in increasing increments (such as doubling the sample volume for each sample). The results for the samples should be parallel to the standard curve. If this does not occur, this may show that something else in the sample (i.e. fats) is interfering with the binding. All samples must also fall on the curve. Some samples were either too low or too high on the curve, meaning we had too much or too little hormone in the sample. If the sample runs on the low end of the curve, you would have a very low optical density or too much hormone in the sample. The opposite is true for samples falling on the higher end of the curve. If you have too much hormone in your sample, dilute the sample. If there is too little hormone, add more volume to the sample when you run it and make up the volume in all other wells with a buffer such as Charcoal stripped serum or phosphate-buffer solution (**PBS**). Both DAb kits had to be diluted to fit all parallelism samples on the curve. However, we were not able to successfully complete parallelism for any EIA with bovine serum because there was increasing optical density with increasing sample volume (interference with the binding). We hypothesized that there is too much fat in the serum samples and the next step was to extract.

Extracting the samples takes everything out of the serum except the hormone in question. We performed an extraction using diethyl ether and used 3H-steroids to calculate extraction efficiency. The use of radioactive material to calculate the efficiency is not necessary, however we chose to use it so we can more calculate the efficiency with more accuracy. The extracted samples were reconstituted in PBS. Because our samples were in a new media, we made new standards with PBS to run the curves and parallelism. We were able to achieve parallelism on both of the DAb kits. However, after two attempts at achieving parallelism with the SAb kits, progress was stopped to minimize costs.

Once we achieved parallelism, we ran the same 9 samples for E2 and P4 on the RIA and DAb kits for comparison. The 9 samples were comprised of the same three time points in one estrous cycle for three different cows. The three time points included: a time point of high P4, when the cows had an active corpus luteum; a time point of high E2, immediately prior to ovulation based on transrectal ovarian ultrasound; and a time point with levels of each hormone in between the other two time points.

The DAb kits were able to estimate results for P4 and E2 in bovine samples. Future work will be done to run statistics and compare the EIA results to the "gold-standard" RIA kits. If comparisons are not different, this study can provide standardization of the techniques required for analysis of these hormones. This will allow accurate interpretation of results from a wide range of research studies conducted within the dairy industry.